PB86-109493

Application of Chemical Frationation/Aquatic Bioassay Procedure to Hazardous Waste Site Monitoring

Acurex Corp., Mountain View, CA

Prepared for

Environmental Monitoring Systems Lab.
Las Vegas, NV

Sep 85

U.S. Department of Commerce
National Technical Information Service
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TECHNICAL REPORT DATA

(Please read Instructions on the reverse before completing)

1. REPORT NO. EPA/600/4-85/059

2.

3. RECIPIENT'S ACCESSION NO. PB86 109493/AS

4. TITLE AND SUBTITLE
APPLICATION OF CHEMICAL FRACTIONATION/AQUATIC BIOASSAY PROCEDURE TO HAZARDOUS WASTE SITE MONITORING

5. REPORT DATE September 1985

6. PERFORMING ORGANIZATION CODE

7. AUTHOR(S) V. Lopez-Avila, W.D. McKenzie, W.W. Sutton, R. Kaminsky, U. Spanagel, T.A. Olsson, and J.H. Taylor

8. PERFORMING ORGANIZATION REPORT NO.

9. PERFORMING ORGANIZATION NAME AND ADDRESS Acurex Corp., Energy & Environmental Div., 555 Clyde Ave., P.O. Box 7555, Mountain View, CA 94039; Bioassay Systems Corp., 225 Wildwood Ave., Woburn, MA 01801; PEDCo Environmental, 1149 Chester Road, Cincinnati, OH 45246; LEMSCo, P.O. Box 15027, Las Vegas, NV 89114

10. PROGRAM ELEMENT NO.

11. CONTRACT/GRANT NO.
68-03-3100 - Acurex task 2
68-02-3173 - PEDCo task 73
68-03-3050 - LEMSCo task 75.01

12. SPONSORING AGENCY NAME AND ADDRESS
Environmental Monitoring Systems Laboratory - LV, NV
Office of Research and Development
U.S. Environmental Protection Agency
Las Vegas, NV 89114

13. TYPE OF REPORT AND PERIOD COVERED

14. SPONSORING AGENCY CODE
EPA/600/07

15. SUPPLEMENTARY NOTES
Project Officer: William W. Sutton

16. ABSTRACT

The chemical fractionation/aquatic bioassay test basically involves biological testing, first using a given collection of leachate, surface water, or liquid waste, and then using fractions and subfractions of the original sample material. The final test result, derived from a compilation of these different bioassay responses, is used to identify bioactive fractions of the original sample material, to assess some of the additive, synergistic and/or antagonistic effects caused by the component waste chemicals, and to provide a preliminary (or screening) hazard evaluation for the aquatic ecosystem. While the procedure has been successfully used for monitoring industrial pollutants, the overall technique was not considered to be ready for use at hazardous waste sites. Results from an initial evaluation indicated that procedural revisions were necessary to improve the chemical fractionation phase. When these procedural revisions had been made, another evaluation was conducted using samples taken from actual hazardous waste sites.

In spite of the complex matrix encountered when using liquid waste material, the fractionation technique was reasonably effective at partitioning the neutral organics. Partitioning of inorganics was also reasonably efficient. However, partitioning of polar organics was not particularly impressive and several of the alcohols and acids were recovered in both inorganic and organic fractions. Further studies will perhaps improve the fractionation efficiency and, therefore, improve the overall procedure's usefulness as a monitoring method.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

18. DISTRIBUTION STATEMENT RELEASE TO PUBLIC	**19. SECURITY CLASS** *(This Report)* UNCLASSIFIED	**21. NO. OF PAGES** 122
	20. SECURITY CLASS *(This page)* UNCLASSIFIED	**22. PRICE**

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS OBSOLETE

PB86-109493

EPA/600/4-85/059
September 1985

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by

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Contract Numbers

68-03-3100 Task 2
68-02-3173 Task 73
68-03-3050 Task 75.01

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Notice

The information in this document has been funded wholly or in part by the United States Environmental Protection Agency under contracts 68-03-3100 Task 2 to Acurex Corporation, 68-02-3173 Task 73 to PEDCo Incorporated, and 68-03-3050 Task 75.01 to Lockheed Engineering and Management Services Company, Incorporated. It has been subject to the Agency's peer and administrative review, and it has been approved for publication as an EPA document.

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Acknowledgements

This method evaluation was conducted by the Environmental Monitoring Systems Laboratory - Las Vegas through a series of extramural contract tasks. These contracts were with 1) PEDCo Environmental, Cincinnati, Ohio, (Contract No. 68-02-3173, task 73); 2) Acurex Corporation, Mountain View, California, (Contract No. 68-03-3100, task 2); and 3) Lockheed Engineering and Management Services Company, Las Vegas, Nevada, (Contract No. 68-03-3050, task 75.01). The chemical fractionation/aquatic bioassay was originally developed (i.e. applied) by G. E. Walsh and R. L. Garnas of the Environmental Research Laboratory - Gulf Breeze. Dr. Garnas also reviewed several phases of the current evaluation and provided constructive suggestions.

Executive Summary

The chemical fractionation/aquatic bioassay test basically involves biological testing, first using a given collection of leachate, surface water, or liquid waste, and then using fractions and subfractions of the original sample material. The final test result, derived from a compilation of these different bioassay responses, is used to identify bioactive fractions of the original sample material, to assess some of the additive, synergistic and/or antagonistic effects caused by the component waste chemicals, and to provide a preliminary (or screening) hazard evaluation for the aquatic ecosystem. While chemical analysis of sample material is not a part of the procedure, a combination of biological test data and chemical analytical data will allow for identification of compounds and groups of compounds that present the greatest environmental hazard.

Skeletonema costatum (saltwater algae), *Selenastrum capricornutum* (freshwater algae), *Mysidopsis bahia* (saltwater crustacean), and *Daphnia magna* (freshwater crustacean) are used as test organisms during the assay. However, the saltwater algae and saltwater crustacean tests are not conducted if an inland location of a particular waste site precludes any possibility of estuary contamination. There is a good data base available for these frequently used biological procedures that indicate assay responsiveness to many pollutant compounds, and some progress has been made toward method standardization. The test species are all of ecological importance to the respective freshwater and estuary environments; they are available commercially, and stock populations or cultures are fairly easy to maintain at the testing laboratory. In spite of the procedure's successful application for monitoring industrial pollutants, the overall technique was not considered to be ready for use at hazardous waste sites. Preliminary testing had indicated some potential problems with the chemical fractionation phase; therefore, an evaluation was conducted using a synthetic (laboratory prepared) waste leachate sample. Since the results from this initial evaluation indicated that procedural revisions were necessary, a series of experiments were conducted to improve the chemical fractionation phase of the overall technique. When these procedural revisions had been made, another evaluation was conducted using samples taken from actual hazardous waste sites.

During each separate chemical fractionation/aquatic bioassay, a portion of the original sample material is first tested for toxicity using the different bioassay procedures. If a toxic response (to include either stimulation or inhibition of the algal populations) does not occur in any of the component assays, no further testing is conducted using this particular waste or leachate collection. If a toxic response does occur for any of the component tests, a second portion of the waste material is then chemically fractionated and each fraction is separately tested using the crustacean and algal assays. The resulting fractions or test samples are the (1) total organics, (2) base/neutrals, (3) organic acids, (4) organic residuals, (5) recombination of 2, 3, and 4, (6) total inorganics, (7) cations, and (8) anions. The overall test result is therefore used to identify bioactive fractions of the leachate or waste collection, to assess some of the additive, synergistic, and/or antagonistic effects caused by the component waste chemicals, and to provide a preliminary (or screening) hazard evaluation for the aquatic environment.

The fractionation procedure can process up to a 2-liter sample, but additional fractionations are frequently necessary before biological testing begins. While replicate fractionation runs are being completed, those fractions and subfractions collected from the initial fractionation are stored at approximately 4°C prior to compositing the respective final samples. When the fractionation phase is complete, each of the final test samples mentioned above should contain a sufficient volume (i.e., volume/concentration) to provide sample material for the four bioassay tests. Obviously, it is better to have an excess of sample material than to discover that there is insufficient material to complete the component assays.

The sample is initially filtered through a 0.45 μ glass fiber filter. The filtrate is then loaded on an XAD-4 column. An XAD-4 and an XAD-8 resin column are used in tandem with the eluate from the XAD-4 resin being passed through the XAD-8 column (addition of XAD-8 resin based on protocol revision). Water (HPLC grade) is added and, after the two column extraction, the aqueous eluate should contain the total inorganic fraction. Organic compounds are eluted from the XAD-4 and XAD-8 resins with acetone and diethylether, respectively.

The total organics fraction is then concentrated (to remove the acetone and diethylether) and extracted with methylene chloride at a pH>12 to isolate the base/neutrals. The remaining aqueous phase is then extracted with methylene chloride at a pH<1 to isolate the acid subfraction. A solvent exchange step (dimethylsulfoxide) completes the fractionation procedure for these subsamples. The remaining aqueous phase (i.e., organic residuals subfraction) is concentrated to dryness and resuspended in dimethylsulfoxide. A proportionate amount of base/neutrals, organic acids, and organic residuals are combined for the recombination fraction. The reconstituted total organics fraction provides a separate sample for biological testing.

The aqueous phase from the initial column separation (XAD-4 and XAD-8 resins) contains the total inorganics fraction. Separate subsamples of the inorganics fraction are then fractionated, using ion exchange resins to provide the respective cation and anions samples. A Dowex 1-X8 column is used to provide the cation subfraction and a Dowex 50 W-18 column is used to provide the anion subfraction. Both columns are eluted with deionized water.

Of the nine separate sample types (i.e., original sample, total organics, base/neutrals, organic acids, organic residuals, recombination of organic subfractions, total inorganics, cations, and anions), only the original material is used for biological testing prior to completing the fractionation phase. If multiple fractionations are required, these would be completed and the resulting fractions composited (with the previous runs) before any additional samples are biologically tested. Eight concentrations or dilutions of each fraction can be tested using each of the four component biological tests, i.e., 0.01, 0.1, 1.0, 10.0, 25, 50, 75, and 100 percent. However, fewer dilutions will frequently provide sufficient information, i.e., 0.01, 1.0, and 100 percent. The 100 percent or original concentration is based on the approximate concentration that existed in the original sample material. Dilution media must be prepared for the respective algal assays, and dilution water must be prepared for the crustacean tests. In some cases, the testing laboratory might not wish to return the sample to the original concentration (i.e., approximate 100 percent concentration based only on volume and not yet based on actual chemical analysis) and would instead test the more concentrated fraction or subfraction.

It should be emphasized that none of the biological assays is conducted as a definitive test in which range finding evaluations precede assays to characterize the concentration response curve. The four component assays are in fact screening procedures being used to test samples of unknown chemical composition, and, for each of the respective assays, a minimum number of test organisms is used. The crustacean assays are both conducted under static conditions where test solutions are not renewed during the testing period. The static test obviously requires less sample material than the flow-through procedures which is sometimes a critical factor when planning assays for a limited amount of leachate fractions and subfractions.

As mentioned above, the current demonstration trials used both hazardous waste site sample material and a synthetic (laboratory prepared) leachate which contained a limited number of compounds, i.e., 2,4-dimethylphenol, cadmium sulfate, 4-chlorohenol, and triethanolamine. The synthetic material was obviously not as complex as the actual hazardous waste and was used as sample material during the first part of the evaluation. Chemical analysis of the resulting fractions and subfractions indicated a need for further testing and revision of the fractionation procedure, i.e., the individual compounds were not efficiently partitioned during fractionation. This need for protocol revision was also reflected in some of the bioassay test data which were, of course, acquired from testing the poorly partitioned fractions. This initial demonstration also indicated the need for some minor revisions to the bioassay portion of the protocol, especially in the instructions for preparing the various fraction samples immediately prior to biological testing (e.g., solvents, nutrient supplements, suggested amounts of sample matrial, number of dilutions, etc.). Several problems were encountered when preparing the laboratory cultures of Skeletonema costatum, and specific protocol revisions have been made for the required salts, metals, and vitamins used in the culture media.

Several preliminary studies were then conducted in an effort to improve the fractionation scheme prior to fractionating the waste material. These preliminary efforts included (1) a pretest fractionation of a waste site sample, (2) an attempt to separately elute the organic acids and the base/neutrals from the XAD-4 resin by sequential elution, (3) a test where phenol and pentachlorophenol concentrations were determined in a modified mass balance study using two different waste sample volumes, (4) a study of compound recovery (at two different spiking concentrations) where fractionations using the XAD-4 resin were compared with fractionations using the XAD-4/XAD-8 resin column sequence, and (5) a brief study which examined potential compound losses during sample concentration.

A complete method protocol for the chemical fractionation/aquatic bioassay procedure is given in the appendix section of this report. It is the most complete version of the procedure and is one that includes the revisions made following the synthetic sample fractionation and separate experiments mentioned above. However, based on the subsequent fractionations of actual hazardous waste site material, additional method revisions, beyond those already incorporated into the procedure, will be required before an efficient fractionation can be consistently achieved, and before the overall technique can be considered for use in an operational monitoring network.

The hazardous waste material used during the evaluation was collected from two different waste sites. While known toxic and carcinogenic compounds were present in the waste material from both sites, these particular sites were selected (one in California and one in Oregon) because of the diversity of chemical compounds known to be present at each location. Material from the different locations was designated as collection A and collection B, respectively.

Collection A contained fairly high concentrations of polar compounds, and, when the revised fractionation procedure was followed, the overall recovery of these polar organics was not as efficient as the observed recovery of the base/neutrals. In addition, those polar compounds which were recovered from the XAD-4 and XAD-8 resins were frequently eluted with the aqueous phase into the total inorganic fraction. This failure to achieve the intended partitioning of component compounds would have caused obvious data interpretation problems had these collection A fractionation samples been biologically tested. Polar organics were also present in the collection B material, but they apparently did not overload the binding capacity of the resins, and consequently the compounds were eluted, as expected, into the total organic fraction.

The current method demonstration, using complex waste site material, has therefore indicated some areas in which the fractionation procedure seems to be fairly effective, but it has also identified other areas where the procedure must be improved. In spite of the complex matrix encountered with both waste collections, the current fractionation technique was reasonably effective at partitioning the neutural organics. Partitioning of inorganics was also reasonably efficient. However, partitioning of polar organics was not particularly impressive and several of the alcohols and acids were recovered in both inorganic and organic fractions. This breakthrough of polar organics was particularly dramatic when the original sample material contained high concentrations of these polar compounds. Based on these results, the chemical fractionations/aquatic bioassay procedure was judged as not being ready for use at actual hazardous waste sites in spite of the fact that the overall procedure continues to be a potentially promising technique. It should be remembered that, under routine method application, the sample material will not have been chemically analyzed prior to conducting the test, and an efficient sample fractionation will be necessary before the resulting biological data can be of maximum benefit. Future studies will perhaps improve the fractionation efficiency, and, therefore, improve the overall procedure's usefulness as a monitoring method.

I. Introduction

Assessing current and potential problems at uncontrolled waste sites has been very difficult. The problem of evaluating complex chemical mixtures rather than specific chemical compounds causes part of the difficulty. Another problem is that toxicity data and environmental transport data are limited for many of the waste compounds, especially those that are byproducts of organic synthesis as opposed to commercial products. Biological monitoring techniques can often be very useful when dealing with such problems, and the biological procedures are particularly effective when combined with chemical analysis of the sample material.

The chemical fractionation/aquatic bioassay procedure basically involves testing samples of hazardous waste (or leachate) as well as chemical fractions and subfractions of the material. A portion of the original waste or leachate sample is first tested for toxicity using the freshwater and marine algal bioassays and the freshwater and marine crustacean bioassays that compose the component parts of this overall test. If a toxic response (to include either stimulation or inhibition of the algal populations) does not occur in any of the component assays, no further testing is conducted using this particular waste collection. If a toxic response does occur for any of the component tests, a second portion of the waste material will then be chemically fractionated and each fraction will be separately tested using the crustacean and algal assays.

Some of the component bioassays have been used, with various modifications, for several years. The overall chemical fractionation/aquatic bioassay procedure was originally developed by Walsh and Garnas at the U.S. Environmental Protection Agency's Environmental Research Laboratory in Gulf Breeze, Florida, and has been successfully used to test many chemically complex industrial effluents (Walsh and Garnas, 1980; Walsh, et al., 1982). In spite of the procedure's success as a monitoring technique for industrial pollutants, the procedure was not considered ready for a single laboratory evaluation (first step in the method validation process), primarily because of incomplete instructions for the chemical fractionation phase, potential problems resulting from the chemical complexity of hazardous waste site samples, and because of apparent problems with the preparation of fraction samples immediately prior to biological testing. While it is assumed that the original sample material will be a liquid, samples collected from hazardous waste sites can include aqueous, organic, and solid phases.

Chemical analysis of sample material is not a part of the chemical fractionation/aquatic bioassay procedure. However, during this method evaluation, many chemical analyses were conducted to assess the effectiveness of the technique. Organic analyses were conducted using computer assisted gas chromatography/mass spectrometry, and the inorganic analyses were conducted using inductively coupled argon plasma spectrometry and atomic absorption spectrometry.

The first step in the method evaluation was to review the existing protocol in order to improve the completeness and clarity of all procedural instructions. A demonstration test of the entire procedure was then conducted using a synthetic (laboratory prepared) waste leachate sample. However, some problems were encountered with the fractionation scheme, and it appeared that procedural revisions to the fractionation phase might be necessary. Actual hazardous waste site samples were then collected from different commercially operated waste sites, and the fractionation phase was again tested using two different hazardous waste site collections.

II. Application of Procedure to Waste Site Monitoring

The demonstration trial used both hazardous waste site sample material and a synthetic (laboratory prepared) leachate which contained a limited number of compounds. The synthetic material was obviously not as complex as the actual hazardous waste and was used as sample material during the first part of the evaluation. The actual hazardous waste site material, collected from two separate waste site locations, presented more of a challenge for the fractionation scheme and was in some ways a more realistic sample material. However, the laboratory prepared material could easily simulate a hazardous waste leachate or a collection of surface water near an uncontrolled waste site.

The fractionation procedure does not necessarily provide an absolute separation of the different compounds, and, depending on the specific chemicals present in the original sample, some portion of the organics may remain in the inorganic fraction, or, in some instances, the base/neutrals might not be completely separated from the organic acids. However, there should be a greater concentration of any particular compound in the expected fraction than there would be in any of the other fractions due to incomplete separation. The total test result for the chemical fractionation/aquatic bioassay provides an overview of toxicity data for a particular sample and shows the component biological test responses relative to other fractions and subfractions of the original waste or leachate collection.

The overall method demonstration resulted in several revisions to the technique. The chemical fractionation scheme was improved, and the revised protocol now includes more complete procedural instructions for many of the fractionation steps. Most of the procedural revisions to the component bioassays were for completeness of instruction and for sample preparation immediately prior to biological testing. (Note: The complete Chemical Fractionation/ Aquatic Bioassay protocol is given in the Appendix. This procedure includes many of the method improvements that have resulted from the current evaluation.)

A. Demonstration Using Laboratory Prepared Leachate

The synthetic waste was composed of only four compounds, i.e., 2,4-dimethylphenol, cadmium sulfate, parachlorophenol, and triethanolamine hydrochloride. Following chemical fractionation, the various fraction and subfraction samples would theoretically contain the compounds shown in Table 1.

1. Chemical fractionation

Approximately 100 liters of the synthetic material were prepared and temporarily stored in polypropylene containers at 4°C. A portion of the material was fractionated according to the initial protocol instructions (figure 1). A total of 29.1 liters of the synthetic waste sample was filtered through a

TABLE 1. LABORATORY PREPARED SAMPLE MATERIAL AND THE RESULTING FRACTIONS AND SUBFRACTIONS ASSUMING COMPLETE SEPARATION DURING FRACTIONATION PROCEDURE

1. Whole Waste Material
   - 2,4-dimethylphenol
   - cadmium sulfate
   - 4-chlorophenol
   - triethanolamine
2. Total Organics Test Sample
   - 2,4-dimethylphenol
   - 4-chlorophenol
   - triethanolamine
3. Base/Neutrals Test Sample
   - triethanolamine
4. Acid Test Sample
   - 4-chlorophenol
   - 2,4-dimethylphenol (poorly extracted)
5. Organics Residuals Test Sample
   - 2,4-dimethylphenol
6. Recombined (Pooled) Samples 3,4 + 5
   - 2,4-dimethylphenol
   - 4-chlorophenol
   - triethanolamine
7. Total Inorganics
   - cadmium sulfate

9. Anion Test Sample
   - sulfate

8. Cation Test Sample
   - cadmium

Note: A fractionation procedure does not necessarily provide an absolute separation of different sample components, and the above fractions represent a theoretical result.

142 mm glass-fiber (nucleopore 20 μm) filter prior to fractionation through the XAD-4 resin column. A flow rate of 8 to 16 ml per minute was maintained during the elution through the filter/XAD-4 resin column apparatus. After collection into a single container, the filtrate was divided into three 9 liter volumes. The remaining 2.1 liters were retained for chemical analysis. One of the 9 liter aliquots was placed in a sealed polypropylene container, labeled as total inorganics fraction, and forwarded for aquatic bioassay testing. The remaining two 9 liter aliquots were further fractionated to obtain the cation and anion subfractions.

The fractionation to obtain the cation subfraction was performed by first adjusting the pH of one of the 9 liter aliquots to pH 10 with 5 ml of 10N sodium hydroxide. The Dowex 1-X8 anion exchange resin (90 g) was then added, and the mixture was mechanically stirred for approximately 24 hours. After it was stirred, the mixture was decanted and filtered to remove the resin. The pH of the filtrate was adjusted to pH 7.0 with hydrochloric acid and placed in a sealed container. During pH adjustment, the color of the filtrate changed from colorless to slightly yellow. The sealed container was labeled cation fraction and forwarded for aquatic testing. The final 9 liter aliquot was also fractionated to obtain the anion subfraction. First, the pH

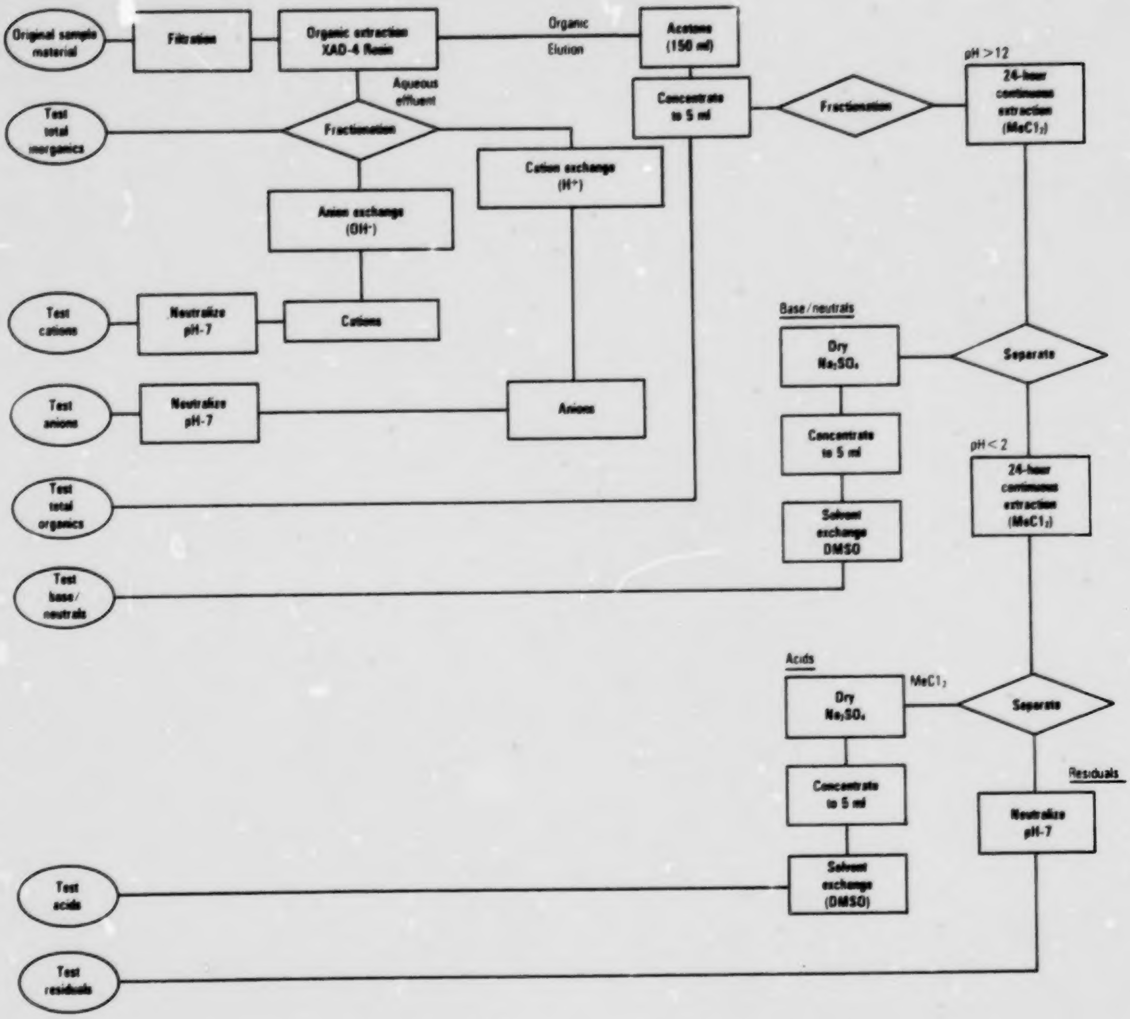
Original sample material
Filtration
Organic extraction XAD-4 Resin
Organic
Elution
Acetone (150 ml)
Aqueous effluent
Test total inorganics
Fractionation
Concentrate to 5 ml
Fractionation
pH >12
24-hour continuous extraction (MeCl$_2$)
Cation exchange (H$^+$)
Anion exchange (OH$^-$)
Test cations
Neutralize pH-7
Cations
Base/neutrals
Dry Na$_2$SO$_4$
Separate
Test anions
Neutralize pH-7
Anions
Concentrate to 5 ml
pH < 2
24-hour continuous extraction (MeCl$_2$)
Test total organics
Solvent exchange DMSO
Test base/neutrals
Acids
MeCl$_2$
Dry Na$_2$SO$_4$
Separate
Residuals
Concentrate to 5 ml
Neutralize pH-7
Test acids
Solvent exchange (DMSO)
Test residuals

Figure 1. Initial fractionation scheme which allowed for the preparation of samples for subsequent biological testing. Recombination sample (recombination of organic subfractions) not shown.

was adjusted to pH 4 with 1.8 ml of concentrated hydrochloric acid. Ninety grams of Dowex 50W-X8 (20-50 mesh) cation exchange resin were then added and the mixture was mechanically stirred for approximately 24 hours. The stirred mixture was then filtered, and the filtrate was neutralized to pH 7.0 with NaOH. The filtrate was placed in a sealed container and labeled as anion fraction. No color change was noted during the pH adjustment. The anion subfraction was retained for bioassay testing. Following elution of the synthetic waste sample, the XAD-4 resin column was aspirated by vacuum to remove the excess water. The XAD-4 column was then eluted with 150 ml of acetone, which was collected in a 300 ml round-bottom flask.

After all the acetone was eluted through the column and collected in the round-bottom flask, the flask was placed on a rotary evaporator and the acetone fraction was concentrated (by rotary evaporation) to approximately 23 ml, which consisted of two liquid phases. The concentrate was then transferred to a 250 ml Erlenmeyer flask, and the round-bottom flask was rinsed with small quantities of water until the two phases were observed to become miscible. The final total volume of this fraction was 900 ml. Based on volume measurements alone, the concentration factor was calculated as follows:

$$\frac{\text{29.1 liters of original sample applied}}{\text{0.9 liter of concentrated sample}} = 32.3$$

The 900 ml was divided into 500 ml, 300 ml, and 100 ml aliquots. The 300 and 100 ml aliquots were labeled as the total organics fraction. The 300 ml aliquot was then sent for bioassay testing, and the 100 ml aliquot was saved for chemical analysis.

The remaining 500 ml aliquot was further fractionated. For removal of the base/neutral organics, the pH of the 500 ml aliquot was adjusted to pH 11 with 10N sodium hydroxide, and the aliquot was transferred to a Hershberg and Wolfe liquid/liquid continuous extraction apparatus (1500 ml capacity). Sufficient methylene chloride and deionized water were added to attain satisfactory operation of the liquid/liquid extraction apparatus, and the extraction was allowed to continue to approximately 24 hours. After 24 hours, the methylene chloride fraction was removed and placed in a 1 liter glass-stoppered bottle and the aqueous fraction was placed in a 1 liter nalgene bottle. Both containers were stored a 5°C.

The containers were allowed to equilibrate to room temperature before further manipulation. The methylene chloride fraction was then passed through a 20 cm column of sodium sulfate (anhydrous) to remove excess water. The aqueous fraction was adjusted to a pH of less than 2 with sulfuric acid and then re-extracted with fresh methylene chloride for 24 hours (as previously described for the base/neutrals subfraction).

The methylene chloride fraction containing the base/neutrals was evaporated at 75°C using a 500 ml Kunderna-Danish evaporator with a 10 ml concentrator tube and with a three-ball evaporator tube. About 18 hours were required to evaporate the fraction to approximately 5 ml, after which the three-ball snyder column was removed, rinsed with 2 to 3 ml of fresh methylene evaporator chloride, and replaced with a two-ball evaporator tube. The rinsing was performed so that the rinses could be collected in the concentrator tube. The two-ball snyder column was then removed and rinsed with 2 to 3 ml of methylene chloride into the concentrator tube. The fraction was then concentrated under a stream of argon at room temperature to approximately 0.25 ml. The concentrate was diluted to 25 ml with dimethyl sulfoxide (DMSO) and placed in a teflon-sealed, amber bottle, which was labeled as base/neutral subfraction and forwarded to the aquatic laboratory for bioassay testing.

The methylene chloride organic acid subfraction was similarly dried and evaporated; however, splattering was observed at 75°C during the final evaporation to 10 ml. The sample was removed from the evaporator and evaporated further under a stream of argon at room temperature to a final volume of 0.25 ml. The concentrate was resuspended to 25 ml with DMSO and transferred to a teflon sealed amber bottle, which was labeled organic acid subfraction and retained for bioassay testing. The remaining 500 ml aqueous fraction was placed in a sealed nalgene bottle labeled organic residuals subfraction and retained for bioassay testing.

Final concentration factors (based only on volume) for the base/neutral, acid, and residual fractions were calculated as follows:

Base/neutral and acid subfractions

$$\frac{500 \text{ ml volume}}{25 \text{ ml volume}} = 20 \text{ fold concentration}$$

$$\frac{500 \text{ ml}}{900 \text{ ml}} = 0.56 \times 20 \times 32.3$$

$$= 362 \text{ concentration factor.}$$

The organic residuals were not further concentrated; therefore, 32.3 was used as the concentration factor.

Portions of the original synthetic material and of the resulting fractions and subfractions were chemically analyzed to verify compound separation during the fractionation process (Table 2). Analyses for triethanolamine were not conducted due to the lack of a suitable analytical procedure. However, the original synthetic material should have contained 134 mg/liter of triethanolamine hydrochloride. It will be noted that relatively high concentrations of chloride and sulfate anions were detected in both cation and anion subfractions. This apparent lack of success with inorganic fractionation was not encountered when actual waste site material was used, and it seems likely that either the sulfate and chloride concentrations reported in the table are not correct or that an error was committed during the fractionation.

2. Aquatic bioassay

Eight samples were tested using the component bioassays, i.e., the original synthetic material, total organic fraction, total inorganic fraction, organic acids subfraction, base/neutrals subfraction, organic residuals subfraction, cation subfraction, and anion subfraction. Due to a limited amount of the respective organic subfractions, it was not possible to test the organic recombination sample that is mentioned in the method procedure. As mentioned before, the four component bioassay test organisms are *Selenastrum capricornutum* (freshwater algae), *Skeletonema costatum* (saltwater diatom), *Daphnia magna* (freshwater crustacean), and *Mysidopsis bahia* (saltwater crustacean).

Fractionation samples were prepared for biological testing by either dissolving appropriate nutrient materials in undiluted waste (i.e., whole waste sample, total inorganic fraction, cation subfraction and anion subfraction) or by dissolving the nutrients in a resolubilized waste fraction or subfraction (i.e., total organic fraction, base/neutral subfraction, acid subfraction, and residue subfraction). These preparations were used at the 100 percent concentration, and serial dilutions were prepared with appropriate (culture) media. The concentration step in the fractionation scheme caused some fractions and subfractions to become highly concentrated. These samples were reconstituted to original (theoretical) concentrations with deionized water.

Daphnia and mysid shrimp were exposed to the various test samples for 48 and 96 hours respectively. The EC$_{50}$ values were calculated by either the Binomial Method, Moving Average Method, or by Profit Analysis. Data analysis/tabulation was performed using a Zenith Model 2-89 microcomputer. A computer program (prepared by C. E. Stephan, EPA) was used for some of the calculations. Algal growth data were tabulated as directed in the method protocol and the results were plotted on probability paper. Final results were presented as 96 hour EC$_{50}$ values (calculated percentage of the sample material that would inhibit growth by 50 percent) and, if applicable, as 96 hour SC$_{20}$ values (calculated percentage of the sample material that would stimulate growth by 20 percent).

Daphnia magna bioassays were conducted at 19°C ± 2° and at an ambient light photoperiod of 16L/8D. Five organisms per container (250 ml Pyrex beakers) and three replicate containers per concentration were used. The organisms were not fed during testing. Test vessels were monitored daily for deaths, and when noted, dead organisms were removed. Dissolved oxygen and pH measurements were made at the beginning and end of testing in a low-, medium-, and high-dose test vessel to determine whether physical changes were taking place in the samples, and to verify whether deaths were related to the toxicity of the sample or to a change in physical conditions in the test vessel. At the end of 48 hours, the number of deaths was recorded. *Mysidopsis bahia* bioassays were conducted at 22° to 25°C and under continuous ambient room-light conditions. Salinity was maintained at 32 to 36 ppt. Five organisms per container (1 liter soft glass jars) and three replicate containers per concentration were used. The mysids were fed approximately 20 (48 hour old) brine shrimp per mysid per day throughout the testing period. Test vessels were monitored daily for deaths, and dead animals were noted and removed. Dissolved oxygen concentration and pH were monitored daily in a low-, medium-, and high-dose test vessel to determine whether physical parameters were fluctuating. At the end of 96 hours, the number of deaths was recorded.

TABLE 2. COMPOSITION OF SYNTHETIC WASTE SAMPLE AND RESULTING FRACTIONS AND SUBFRACTIONS BASED ON ACTUAL CHEMICAL ANALYSIS

Type of Analysis	Original Waste Material (Synthetic)	Total Organics	Organic Acids	Base/ Neutrals	Organic Residuals	Total Inorganics	Cation Sample	Anion Sample	Deionized Water
2,4-dimethyl-phenol by GC/MS, µg/l (except as noted)	2200	9100	84 µg/ml (DMSO)	4800 (DMSO)	<10	81	-	-	<10
p-chloro-phenol by GC/MS, µg/l (except as noted)	4900	>9000	3200 µg/ml (DMSO)	4200 (DMSO)	<10	<10	-	-	<10
triethanol-amine	NA	NA	NA	NA	NA	NA	-	-	NA
analysis for additional organic compounds by GC/MS, µg/l	-	<10	-	<10 (DMSO)	<10	<10	-	-	<10
Cadmium by AA, µg/l	210	-	-	-	-	-	5.0	-	-
Sulfate, mg/l	0.23	-	-	-	-	0.45	0.36	0.64	0.2
Chloride, mg/l	24.8	-	-	-	-	67.2	219	142	1.0

NOTE: The original synthetic material should have contained approximately 134 mg/liter of triethanolamine hydrochloride. However, analysis for triethanolamine was not conducted due to the lack of a suitable analytical procedure. No explanation is given for the respective sulfate and chloride concentrations in both cation and anion subfractions. This apparent lack of success with inorganic fractionation was not encountered when actual waste site material was used, and it seems likely that the sulfate and chloride concentrations reported above are not correct or that an error was committed during the fractionation.

Skeletonema costatum bioassays were conducted at 20°C ± 2° under a photoperiod of 14L/10D and at a light intensity of 450 ± 45 footcandles. Each test vessel (125 ml Erlenmeyer flask) was inoculated with approximately 8.0 x 10^4 cells per ml in a total volume of 25 ml. Flasks were incubated for a period of 96 hours and then immediately fixed in Lugol's fixative. Cell counts were performed on American Optical Improved Neubauer Hemacytometers (0.1 mm deep). *Selenastrum capricornutum* bioassays were conducted at 24°C ± 2° under continuous light conditions and at a light intensity of 450 ± 45 footcandles. Each test vessel (125 ml Erlenmeyer flask) contained a total volume of 25 ml and was inoculated with approximately 2 x 10^4 cells per ml. Flasks were incubated for a period of 96 hours and then immediately fixed in Lugol's fixative to await cell enumeration. As before, cells were counted on an American Optical Improved Neubauer 0.1-mm-deep Hemacytometer.

3. Overall test result

As stated above, the freshwater green alga *Selenastrum capricornutum* and the saltwater diatom *Skeletonema costatum* are used as test species for the respective algal assays. Eight nutrient supplemented samples (each containing a different concentration of the particular waste fraction or subfraction) and the respective control samples are each inoculated with 1 ml of 6 to 8 day old stock algal culture. Three replicates are conducted for each of the eight test samples and for each control. The final assay results are presented as either the EC_{50} (calculated concentration that will inhibit algal growth by 50 percent) or as the SC_{20} (calculated concentration that will stimulate algal growth by 20 percent). The overall chemical fractionation/aquatic bioassay test result for the laboratory prepared waste material is shown in Table 3. This same information is presented again in Table 4 using the somewhat arbitrarily assigned toxicity categories that are defined in Table 5. Stimulation and inhibition test responses were respectively noted for the algal growth assays, and all four of the biological tests were responsive to various fractions and subfractions. The mysid shrimp is apparently the most sensitive bioassay test organism for this particular sample of laboratory prepared material. Stimulation of algal growth was noted for some samples when using *Skeletonema costatum*, and growth inhibition was noted for both algal assays using various other fractions and subfractions. Toxic responses were also noted for several samples using the crustacean assays and were particularly dramatic for the total organic fraction.

Skeletonema costatum test responses using samples of whole waste, total inorganics, anions, cations, total organics, base/neutrals, organic acids, and organic residues are shown in figures 2 through 10. *Selenastrum capricornutum* test responses using samples of whole waste, total inorganics, total organics, base/neutrals, organic acids, and organic residues are shown in figures 11 through 16. The demonstration trial resulted in some improvements to the bioassay portion of the method protocol, primarily in the area of completeness of protocol instructions, required assay controls, suggested minimum volume of original waste sample, and in the instructions for preparing various samples immediately prior to biological testing (e.g., solvents, nutrient supplements, suggested amounts of sample material, number of dilutions, etc.). Several problems were encountered when preparing the laboratory cultures of *Skeletonema costatum*, and specific protocol revisions were made for the required salts, metals, and vitamins used in the culture medium. Instructions for the freshwater algal assay and for both crustacean assays appeared to be more complete, and fewer protocol revisions were required. Improvements to the chemical fractionation portion of the protocol resulted primarily from a subsequently conducted fractionation of actual waste site material.

The protocol directions for the use of positive controls still needs improvement, in that more information should be provided as to which compounds can be used and at which respective concentrations. Zinc chloride was used with the algal tests during this method demonstration, and sodium dodecyl sulfate was used with the crustacean assays. The sodium dodecyl sulfate consistently produced a toxic test response for the crustacean assays (EC_{50} concentration between 10 and 20 μg/ml. The freshwater algae were also consistently inhibited at 20 μg/ml of zinc, but growth of the saltwater diatom was not inhibited at zinc concentrations up to 640 μg/ml. Zinc chloride may not be the best choice as a positive control for the saltwater algae, and a positive control still needs to be selected for the algal growth stimulation response of both algal assays.

TABLE 3. CHEMICAL FRACTIONATION/AQUATIC BIOASSAY RESULT FOR THE METHOD DEMONSTRATION TEST USING THE SYNTHETIC WASTE/LEACHATE SAMPLE

	Saltwater			Freshwater		
	S. costatum		*M. bahia*	*S. capricornutum*		*D. magna*
Sample	SC_{20}	EC_{50}	EC_{50}	SC_{20}	EC_{50}	EC_{50}
Whole waste		6.5	0.8		15.1	15.8
Organic fraction		10.4	2.8		19.3	3.1
Acid subfraction		15.8	10.5		30.5	5.1
Base/neutral subfraction		63.3	15.8		84.6	13.3
Residual subfraction	0.3	c	c		2.3	c
Recombination of organic subfractions		d	d		d	d
Inorganic fraction		16.9	5.6		10.5	0.3
Cation subfraction	64.0	d	e		d	f
Anion subfraction	5.6	e	11.2		f	22.2

[a] SC_{20} = Calculated concentration that will stimulate algal growth by 20 percent.
EC_{50} = Calculated concentration that will inhibit algal growth by 50 percent (or that will produce a toxic response in the crustacean tests).

[b] Interpretation of results is complicated because of poor compound partitioning which occurred during the fractionation phase.

[c] Test response indicates no toxic effect.

[d] Insufficient sample material to conduct the bioassay (additional fractionations should have been conducted before any of the biological tests were started).

[e] EC_{50} value greater than the 100 percent concentrations.

[f] No relationship between sample dilution and test response was evident during the sample assay.

TABLE 4. RELATIVE TOXICITY CATEGORIES USED AS AN ALTERNATE APPROACH FOR PRESENTING SYNTHETIC SAMPLE TEST RESULT

Sample	Saltwater		Freshwater	
	S. costatum	M. bahia	S. capricornutum	D. magna
Whole waste	High	High	High	High
Organic fraction	High	High	High	High
Acid subfraction	High	High	Moderate	High
Base/neutral subfraction	Moderate	High	Low	High
Residual subfraction	a	a	High	a
Recombination of organic subfractions	b	b	b	b
Inorganic fraction	High	High	High	High
Cation subfraction	a	d	b	c
Anion subfraction	a	High	c	Moderate

[a] Test response indicates no toxic effect.

[b] Insufficient sample material to conduct the bioassay (additional fractionations should have been conducted before any of the biological tests were started).

[c] No relationship between sample dilution and test response was evident during the sample assay.

[d] EC_{50} value greater than the 100 percent concentration.

It should be remembered that none of the four assays are conducted as definitive tests in which range finding evaluations precede assays to characterize the concentration response curve. A minimum number of test organisms are used in a static test situation in which test solutions are not renewed during the testing period. Furthermore, a single chemical fractionation will rarely result in complete compound separations into the various fractions and subfractions. Fractionation efficiency will be based on the specific compounds present in the original waste sample. In spite of these known limitations, the overall screening technique does provide relative toxicity information that can indicate the bioactive fractions as well as some of the potential additive,

TABLE 5. RELATIVE TOXICITY CATEGORIES

				Range LC_{50} or EC_{50} concentration (%)			
Assay	Activity Measured	MAD[a]	Units	High	Moderate	Low	Not detectable
Freshwater invertebrate	48-h EC_{50}[b] (immobilization)	100	Percent	0-20	20-75	75-100	ND[c] at 100
Freshwater algae	96-h EC_{50} (growth inhibition)	100	Percent	0-20	20-75	75-100	ND at 100
Marine invertebrate	96-h EC_{50} (immobilization)	100	Percent	0-20	20-75	75-100	ND at 100
Marine algae	96-h EC_{50} (immobilization)	100	Percent	0-20	20-75	75-100	ND at 100

[a] MAD = Maximum applicable dose.

[b] EC_{50} = Calculated concentration expected to produce effect in 50 percent of population.

[c] ND = Not detectable.

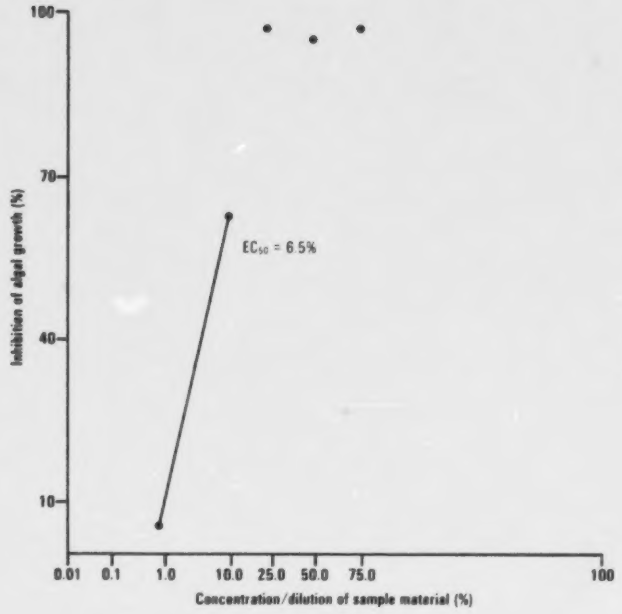

Figure 2. Skeletonema costatum test response to samples of whole waste (laboratory prepared).

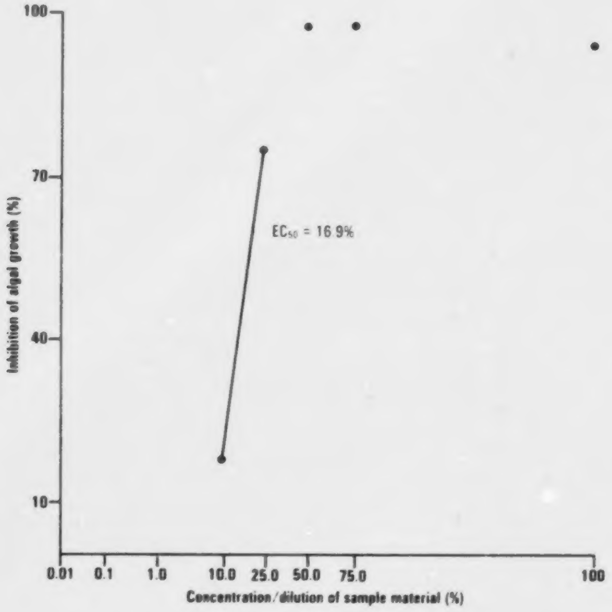

Figure 3. Skeletonema costatum test response to samples of total inorganics.

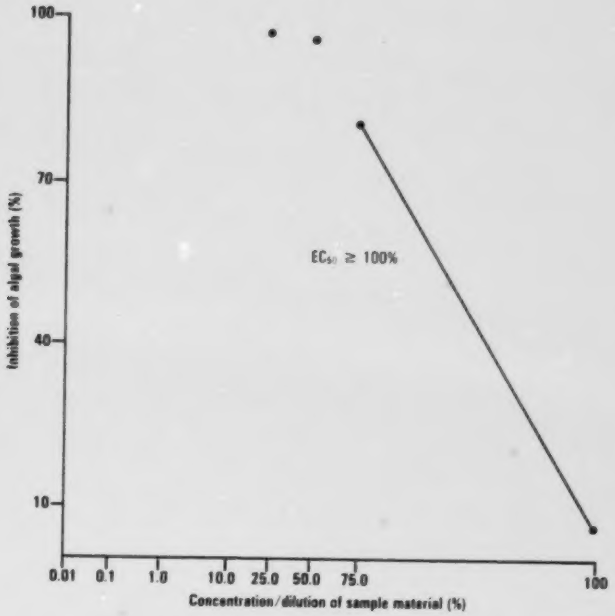

Figure 4. Skeletonema costatum test response to samples of the anion subfraction (EC_{50}).

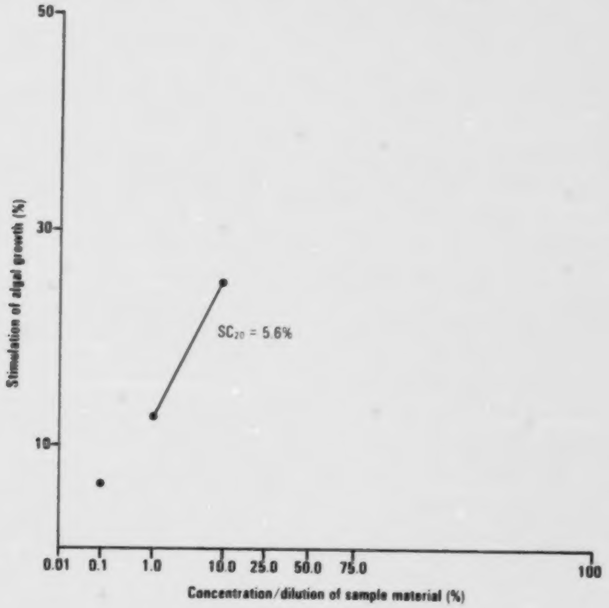

Figure 5. Skeletonema costatum test response to samples of the anion subfraction (SC_{20}).

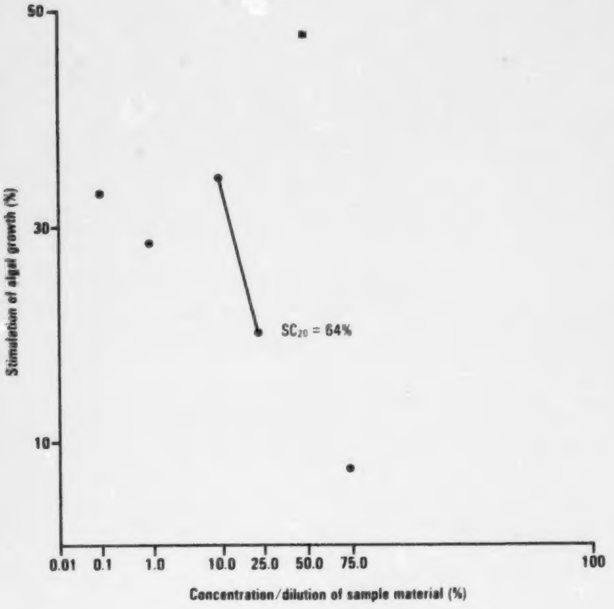

Figure 6. *Skeletonema costatum* test response to samples of the cation subfraction (SC_{20}).

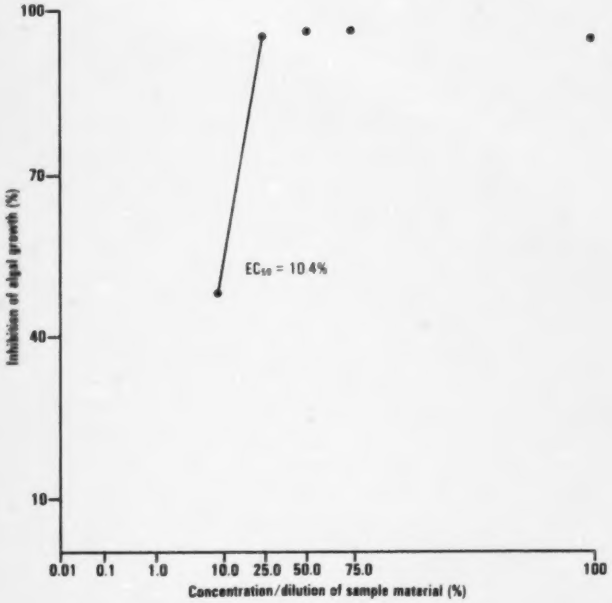

Figure 7. *Skeletonema costatum* test response to samples of total organics.

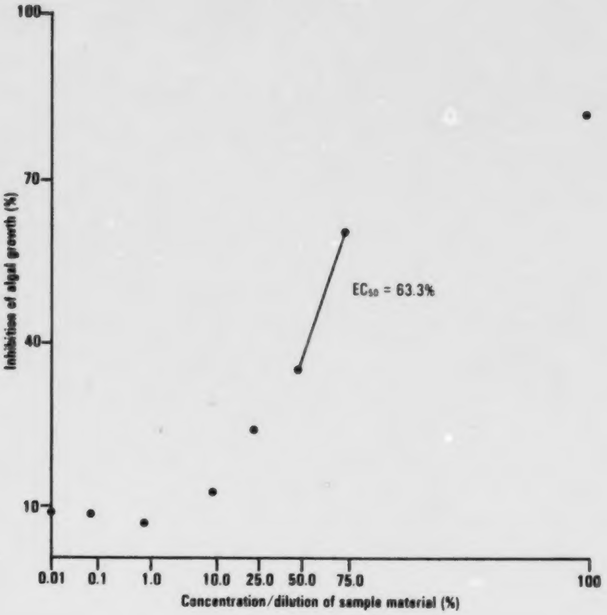

Figure 8. Skeletonema costatum test response to samples of the base/neutrals subfraction.

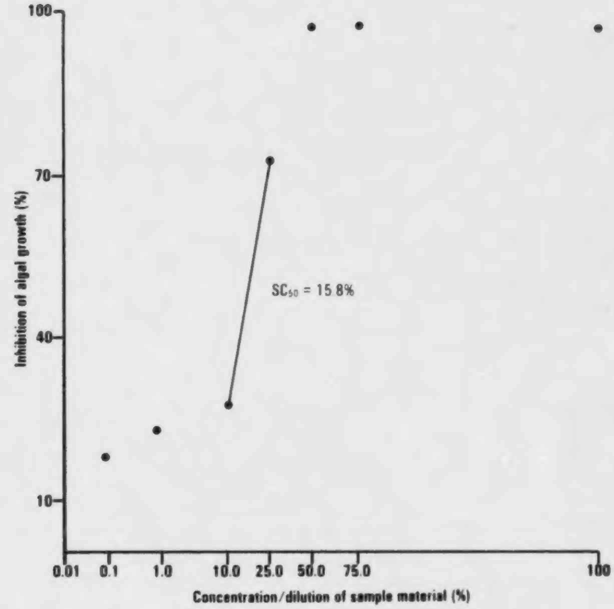

Figure 9. Skeletonema costatum test response to samples of the organic acid subfraction.

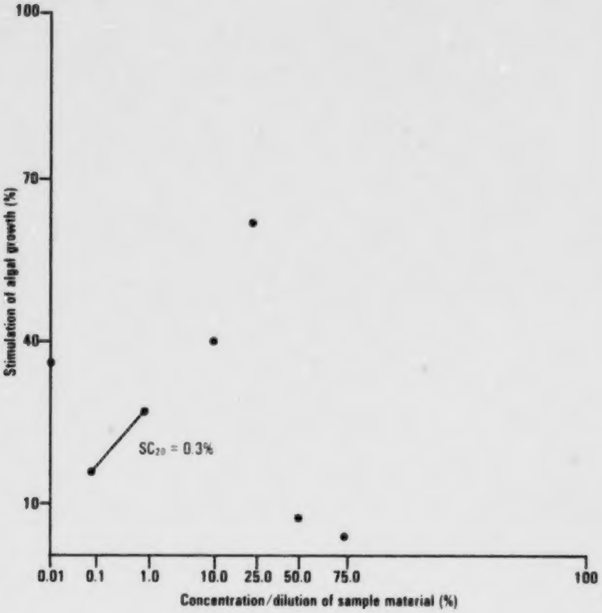

Figure 10. *Skeletonema costatum* test response to samples of the organic residue subfraction (SC$_{20}$).

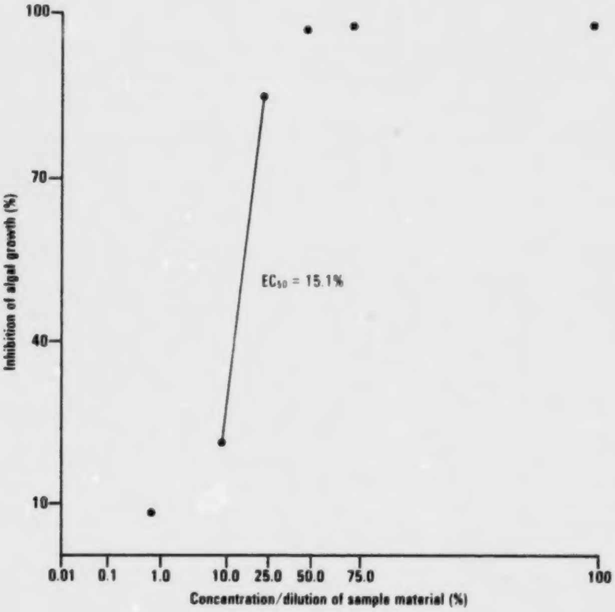

Figure 11. *Selenastrum capricornutum* test response to samples of the whole waste (laboratory prepared).

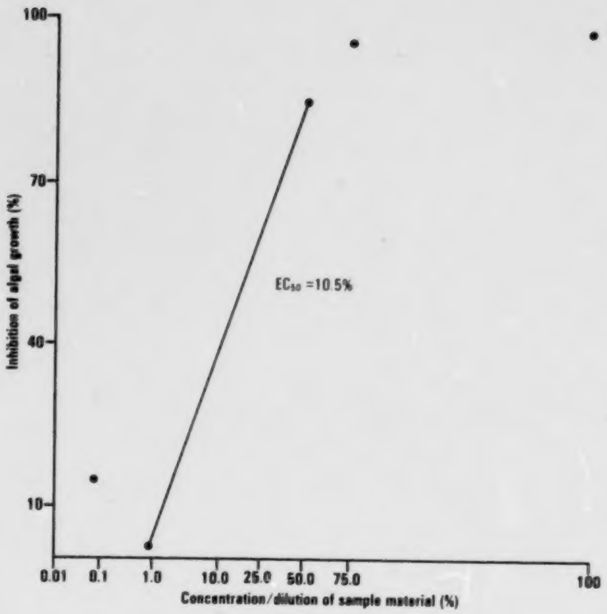

Figure 12. *Selenastrum capricornutum* test response to samples of the total inorganic fraction.

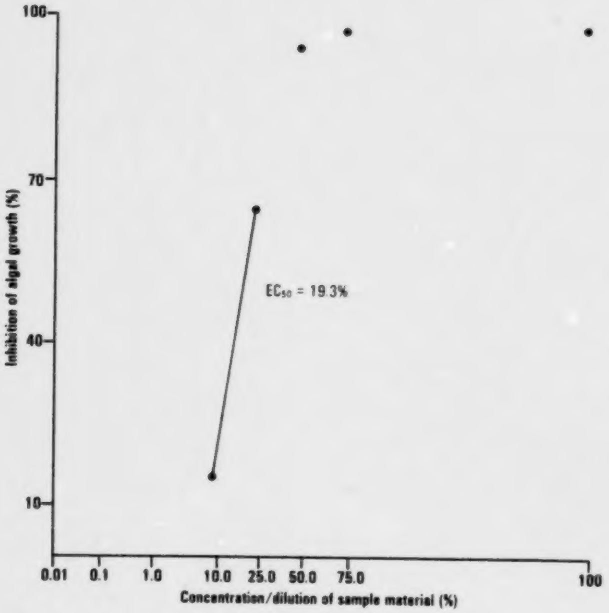

Figure 13. *Selenastrum capricornutum* test response to samples of the total organic fraction.

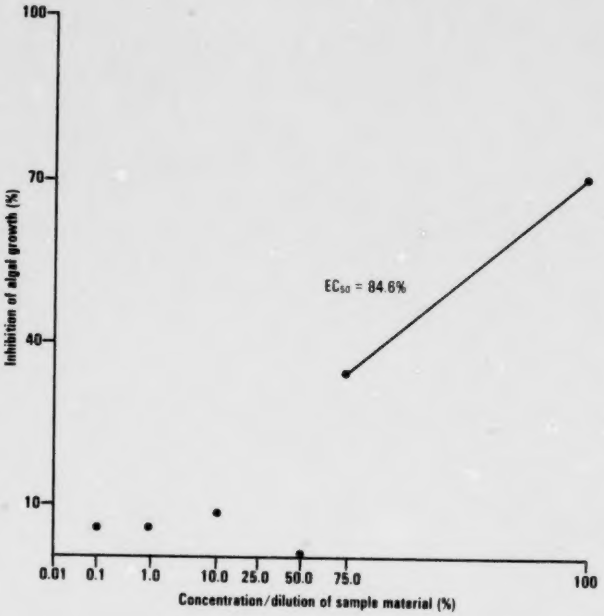

Figure 14. *Selenastrum capricornutum* test response to samples of the base/neutrals subfraction.

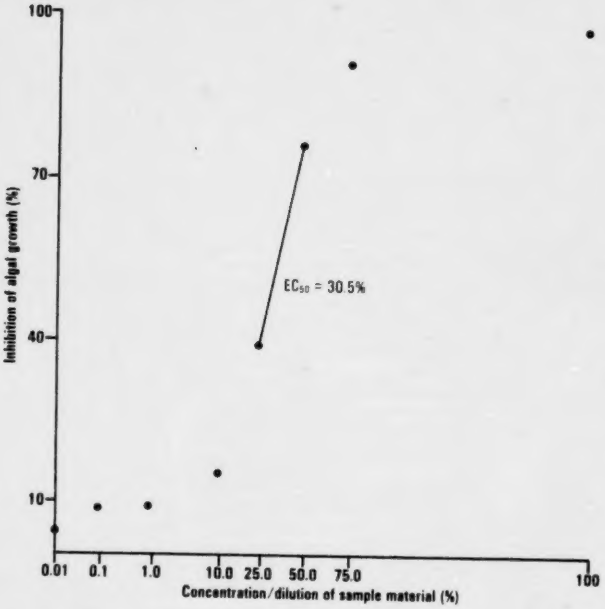

Figure 15. *Selenastrum capricornutum* test response to samples of the organic acid subfraction.

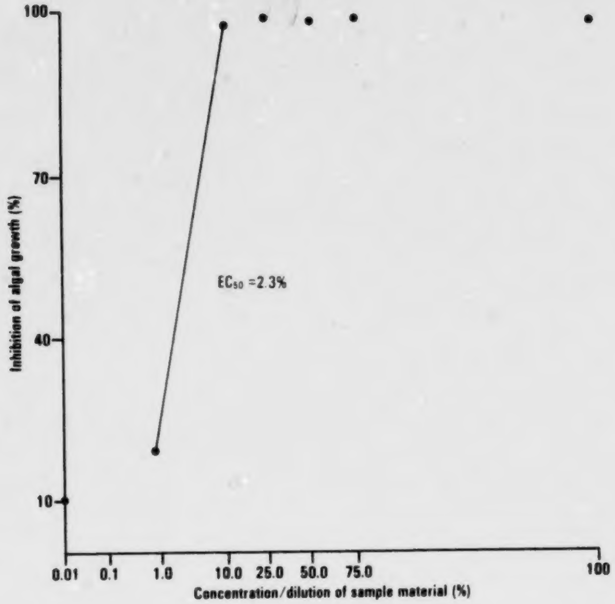

Figure 16. *Selenastrum capricornutum* test response to samples of the organic residue subfraction.

synergistic and/or antagonistic effects caused by the component chemicals. The procedure also provides a preliminary hazard evaluation for the aquatic ecosystem. If a subsequent decision is made to chemically analyze the fractions, subfractions, and original waste material, the resulting combination of biological and chemical data will allow for the identification of compounds and groups of compounds that present the environmental hazard.

B. Demonstration Using Actual Waste Site Material

Actual hazardous waste was collected from two different waste sites. Material from the different locations has been designated as collection A and collection B, respectively. While known toxic and carcinogenic compounds are present in the waste material from both sites, these particular sites were selected (one in California and one in Oregon) because of the diversity of chemical compounds known to be present at each location. The same collection procedure was followed at both locations in that samples collected from the lagoons were composited in a large container, mechanically mixed, and aliquoted into separate smaller containers at time of collection.

At various times during the procedure evaluation, both of the original whole waste samples were chemically analyzed as were the resulting fraction and subfraction samples. Aliquots of the whole waste and of the fraction and subfraction samples were also retained for potential biological testing.

1. Tests conducted to improve fractionation

Several preliminary fractionation trials and associated chemical analyses preceded fractionation of the two separate waste site samples. These preliminary studies included (1) an initial fractionation of the collection A waste sample (using a small sample volume), (2) an attempt to separately elute the organic acids and the base/neutrals from the XAD-4 resin by sequential elution, (3) a test where phenol and pentachlorophenol concentrations were determined in a modified mass balance study using two different waste sample volumes (Tables 6 and 7), (4) a study of compound recovery (at two different spiking concentrations) where fractionations using the XAD-4 resin were compared with fractionations using the XAD-4/XAD-8 resin column sequence (Tables 8 and 9), and (5) a brief study which examined potential compound losses during sample concentration (Table 10).

When attempting the above mentioned single column separations, most of the compounds eluted in the base/neutrals fraction and consequently the method protocol was not modified to include this procedure. In the modified mass balance study, pentachlorophenol (which is a stronger acid than phenol) was not effectively retained on the resin, since some of the molecules were in the ionized form and breakthrough probably occurred. However, during fractionation of the total organics, pentachlorophenol's distribution was more predictable than was the phenol distribution.

When the sequential resin columns were tested, the XAD-8 resin retained some of the compounds that were breaking through the XAD-4 resin (e.g., phenol, dimethylphenol, butoxy-ethoxy-ethanol, tetrachlorophenol, pentap chlorophenol, and methylene-bis-phenol). In spite of some improvement, compounds with a water solubility range in grams/liter (e.g., benzoic acid, hydroxybenzaldehyde, hydroxymethoxybenzaldehyde, hydroxyphenylethanone, etc.) will not be retained by either resin, and will ultimately be present in the inorganic fraction rather than in the total organics fraction.

2. Fractionation of collection A

Approximately 2 liters of collection A waste material were fractionated according to the method procedure (see Appendix). A diagram of the fractionation scheme is given in Figure 17 which also indicates the nine separate samples which would ultimately be tested using the biological assay procedures. The volume of original collection A waste material, the volume of each resulting fraction and subfraction, the volume of material available for biological testing, the volume of material available for potential chemical analysis, and an approximate concentration factor (relative to the concentration of compounds in the original whole waste sample) for each fraction and subfraction are given in Table 11.

a. sample preparation

An attempt was made to filter the thick, dark-brown waste sample through a 0.45-μm filter, but this was unsuccessful. The filter clogged within a few minutes and the flow could not be restored either by increasing the head pressure or by deionized water. To remove some of the solids, the

TABLE 6. PRELIMINARY FRACTIONATION OF COLLECTION A WASTE SAMPLE USING DIFFERENT AMOUNTS OF THE WASTE SITE MATERIAL

	Fractionation Conducted Using 95 ml Sample			Fractionation Conducted Using One Liter Sample		
Compound	Total Organics	Base/ Neutrals	Organic Acids	Total Organics	Base/ Neutrals	Organic Acids
phenol	+	+	+	+		+
butoxy-ethoxy-ethanol	+	+	+	+	+	+
hydroxy-methoxy-benzaldehyde	+		+	+		+
hydroxy-methoxy-phenylethanone	+		+	+		+
pentachlorophenol	+		+	+		+
anthracene-dione	+	+		+	+	
tetrachlorophenol (2 isomers)	+		+	+		+
methylene-bis-phenol			+	+		+
benzenemethanol	+	+	+	+	+	

or by diluting a portion of the sample with deionized water. To remove some of the solids, the sample was passed through a precleaned, stainless steel filter. This procedure was also unsuccessful. Throughout the fractionation process, problems such as clogging of resin columns and formation of viscous solutions during the concentration steps were encountered. The fine particulate content of the sample was apparently causing some of the problems. These problems would be reduced if the fine particulates were removed from the sample. It was also observed that when the sample and its aqueous fractions were stored at <4°C, a crystalline mass was formed. This suggested that the material concentrations were approaching saturation. (Note: Separate chemical procedures concerned with attempts to dissolve the solid material or to grind the particles to an even smaller particle size were not used for either of the waste site samples fractionated during the current evaluation.)

Approximately 2.5 liters of sample were passed through a precleaned, stainless steel filter. A 2-liter portion was used for fractionation; 400 ml were set aside for bioassay testing, and 50 ml were used for chemical analysis. The filtrate was designated as sample 1 (first sample used in bioassay testing scheme).

TABLE 7. MODIFIED MASS BALANCE STUDY WHERE AMOUNTS OF PHENOL AND PENTACHLOROPHENOL WERE DETERMINED IN A WASTE SAMPLE AND IN THE RESULTING FRACTIONS AND SUBFRACTIONS (Results given in μg using two sample sizes of waste site collection A)

	Amount Recovered (μg) Units	
	Phenol	Pentachlorophenol
Starting Waste Material (95 ml)	15,800	15,800
Total Organics	22,100	6,160
Base/Neutrals	190	<100
Organic Acids	11,500	5,200
Starting Waste Material (1,000 ml)	166,000	166,000
Total Organics	177,000	59,600
Base/Neutrals	64,600	<70
Organic Acids	95,900	35,700
Organic Residuals	<50	<50

b. separation of inorganics (sample 7) and organics (sample 2)

The 2-liter portion of the filtered waste sample was passed through an XAD-4 resin column (6 cm ID x 100 cm height) at a flowrate of 15 ml/min. The column clogged after 1,500 ml of eluate were collected and the flowrate was restored by using a slight vacuum. After all of the sample passed through the resin bed, the resin was washed with 15 liters of deionized water. The disappearance of color in the eluate was used to indicate removal of water soluble material from the column. The eluate was initially opaque and dark brown; after washing the resin, the eluate was transparent but still yellow and the resin was a dark brown. The aqueous eluate was then passed through an XAD-8 resin column (6 cm ID x 36 cm height). The elution rate varied from 10 to 20 ml/min. After 4 liters of eluate were collected, the XAD-8 resin column clogged. The flow was restored by stirring the column from the bottom with a steel wire. The total volume of aqueous eluate collected from the XAD-8 column was 24 liters. The eluate was then concentrated on a hotplate to 5.2 liters, thoroughly mixed, and subsampled for bioassay testing (1.3 liters), chemical analysis (75 ml), and further fractionation (3.6 liters). This fraction was designated as fraction 7 and contained all inorganic components. The sample for bioassay testing was further concentrated to 500 ml to achieve approximately the same concentration of inorganics as in the original waste sample.

c. separation of total organic fraction (sample 2)

The XAD-4 resin was eluted with 5 liters of acetone at 20 ml/min until the eluate (initially dark brown) was colorless. The XAD-8 resin was eluted with 3 liters of diethyl ether until the eluate was clear. The

TABLE 8. PRELIMINARY STUDY OF COMPOUND RECOVERY WHERE FRACTIONATIONS USING THE XAD-4 RESIN ARE COMPARED WITH FRACTIONATIONS USING BOTH XAD-4 AND XAD-8 RESIN COLUMNS (Results Presented as a Percentage of the Added Low Level Spike)

		Recovery from Spiked Deionized Water		Recovery from Spiked Hazardous Waste Sample[a]	
Compound	Spike Level (mg/l)	XAD-4	XAD-4/ XAD-8	XAD-4	XAD-4/ XAD-8
naphthalene	1.10	46	80	4	33
pyrene	1.59	20	58	40	37
2,4-dinitrotoluene	1.43	52	67	36	53
nitrobenzene	4.17	38	80	2	33
pentachlorophenol	1.52	4	1	b	b
phenol	1.73	66	5	b	b
benzoic acid	0.97	40	ND	4	ND
aniline	4.23	74	66	44	ND
benzidine	0.54	12	74	14	45
quinoline	1.20	46	82	10	39
1,2,4-trichlorobenzene	0.73	20	50	8	22
hexachlorobenzene	1.06	23	62	8	10
2,4-dimethylphenol	3.23	65	107	2	20
stearic acid	1.08	<1	<1	39	31

[a] Corrections have been made for the presence of these compounds in the original waste sample (except as noted in b).

[b] Due to relatively high concentrations of these compounds in the original unspiked waste sample, the added spike could not be recovered.

ND - Nondetectable

TABLE 9. PRELIMINARY STUDY OF COMPOUND RECOVERY WHERE FRACTIONATIONS USING THE XAD-4 RESIN ARE COMPARED WITH FRACTIONATIONS USING BOTH XAD-4 AND XAD-8 RESIN COLUMNS (Results Presented as a Percentage of the Added High Level Spike)

Compound	Spike Level (mg/l)	Recovery from Spiked Distilled Water		Recovery from Spiked Hazardous Waste Sample[a]	
		XAD-4	XAD-4/ XAD-8	XAD-4	XAD-4/ XAD-8
naphthalene	200	2	<1	11	7
pyrene	200	105	103	100	100
2,4-dinitrotoluene	200	32	21	22	26
nitrobenzene	231	29	27	37	7
pentachlorophenol	200	100	71	71	73
phenol	200	37	65	41	77
benzoic acid	200	33	58	ND	ND
aniline	205	35	19	43	26
1,2,4-trichlorobenzene	291	11	2	17	trace
hexachlorobenzene	200	30	25	23	50
2,4-dimethylphenol	197	44	29	21	10

[a] Corrections have been made for the presence of these compounds in the original waste sample.

ND - Nondetectable

ether eluate contained a dark-colored aqueous layer which was separated and composited with the total inorganic fraction before concentration. The ether eluate from the XAD-8 column was combined with the acetone eluate from the XAD-4 column. Since the acetone and ether eluates contained a substantial amount of water which would interfere later with the methylene chloride extraction, some of the water and the two solvents were removed on a rotary evaporator at 40°C. The remaining aqueous solution (2.1 liters) was designated as fraction 2. A 1.5-liter aliquot of this solution was used for base/neutral and acid extractions; 200 ml were used for GC/MS analysis, and 400 ml were set aside for bioassay testing.

TABLE 10. PRELIMINARY STUDY OF FRACTIONATION PROCEDURE WHICH EXAMINED POTENTIAL COMPOUND LOSSES DURING SAMPLE CONCENTRATION (Results Expressed as Percentage of Spike Recovered in all Organic Subfractions i.e., Base/Neutrals, Organic Acids, and Organic Residuals)

Compound	Recovery from Acetone by Kuderna-Danish Evaporation	Recovery from Aqueous Phase by Extraction with Methylene Chloride
aniline	86	72
phenol	55	55
nitrobenzene	91	100
2,4-dimethylphenol	75	65
1,2,4-trichlorobenzene	74	100
naphthalene	79	96
benzoic acid	75	94
2,6-dinitrotoluene	77	93
hexachlorobenzene	79	100
pentachlorophenol	97	48
pyrene	94	65

d. separation of cations (sample 8) and anions (sample 9)

The total inorganics fraction (sample 7) was divided into two equal aliquots and further fractionated as follows. One aliquot was passed through a Bio Rad AG 1-X8 anion-exchange resin to generate the cations. The other aliquot was passed through a Bio Rad 50W-X8 cation-exchange resin to generate the anions. The amount of ion-exchange resin required in this case was established from the conductivity measurements in Table 12 and the data given by Pellizzari (1983). Table 2 shows the suggested and actual conductivity values for the cation and anion exchange columns.

1) cations

Prior to use, the Bio Rad AG 1-X8 anion exchange resin was conditioned by continuous Soxhlet extraction with methanol for 24 hours, followed by washing with 5 liters of 1N NaOH. The excess NaOH was washed from the resin with about 10 liters of deionized water until the pH was 7. The

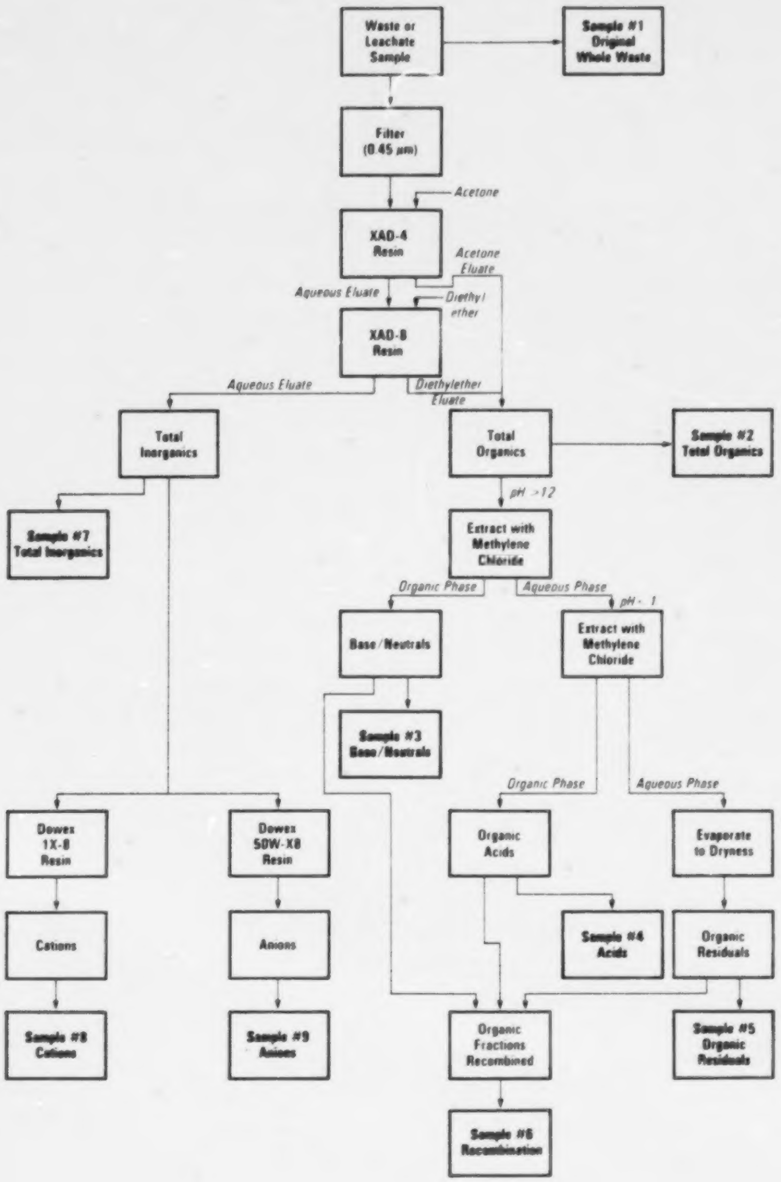
Waste or Leachate Sample
Sample #1 Original Whole Waste
Filter (0.45 μm)
Acetone
XAD-4 Resin
Acetone Eluate
Aqueous Eluate
Diethyl ether
XAD-8 Resin
Aqueous Eluate
Diethylether Eluate
Total Inorganics
Total Organics
Sample #2 Total Organics
Sample #7 Total Inorganics
pH >12
Extract with Methylene Chloride
Organic Phase
Aqueous Phase
pH ~ 1
Base/Neutrals
Extract with Methylene Chloride
Sample #3 Base/Neutrals
Organic Phase
Aqueous Phase
Dowex 1X-8 Resin
Dowex 50W-X8 Resin
Organic Acids
Evaporate to Dryness
Cations
Anions
Sample #4 Acids
Organic Residuals
Sample #8 Cations
Sample #9 Anions
Organic Fractions Recombined
Sample #5 Organic Residuals
Sample #6 Recombination

Figure 17. Chemical fractionation phase of the chemical fractionation/ aquatic bioassay procedure. This fractionation scheme was used to fractionate the collection A waste material.

TABLE 11. RESULTING FRACTION AND SUBFRACTION SAMPLE VOLUMES AND RELATIVE SAMPLE CONCENTRATIONS FOR COLLECTION A WASTE MATERIAL USING THE CHEMICAL FRACTIONATION/AQUATIC BIOASSAY PROCEDURE

	Fraction Processing		Fraction Collection		Fraction Disposition			
Fraction[a]	Process Volume (ml)	Concentration Factor[b]	Collected Volume (ml)	Concentration Factor[b]	Fractionation Volume (ml)	Bioassay Testing Volume (ml)	Concentration Factor[b]	Analysis Volume (ml
1) Filtered waste sample (aqueous)	2,500	1.0	2,500	1.0	2,000	400	1.0	50
2) Total organics (aqueous)	2,100	1.0	2,100	1.0	1,500	400	1.0	200
3) Base/neutrals (DMSO)[c]	1,500	1.0	15	100.0	--	15	95.0	1
4) Acids (DMSO)	1,500	1.0	20	75	--	20	71.0	1
5) Residuals (aqueous)	1,000	1.5	1,000	1.5	--	800	1.4	200
7) Total inorganics (aqueous)	5,200	0.385	3,800	0.4	3,550	1,300[d]	1.0	75
8) Cations (aqueous)	1,900	0.385	600	1.2	--	500	1.2	100
9) Anions (aqueous)	1,650	0.385	1,700	0.4	--	650	0.4	200

[a] For this demonstration trial, the original waste material was initially filtered as opposed to subsampling the unfiltered waste for use as sample 1. Relative concentration factors are therefore based on the filtered waste sample. The recombination of organic subfractions (sample 6) was not prepared during this phase of the demonstration trial.

[b] Concentration relative to filtered waste material.

[c] Dimethylsulfoxide

[d] The volume retained for bioassay test was obtained by concentrating 1,300 ml to 500 ml.

TABLE 12. CONDUCTIVITY DATA AND RESIN VOLUMES FOR THE SEPARATION OF WASTE COLLECTION A CATION AND ANION SUBFRACTIONS

	Conductivity (μmhos/cm)	Resin Volume (ml)	Sample Volume (ml)	Resin to Sample Ratio
Suggested Values	30,000 to 60,000	10	5	2
Actual Value for Collection A Sample	614,000	4,100[a]	1,900[a]	2.16[a]
		3,600[b]	1,650[b]	2.18[b]

[a] Anion exchange resin
[b] Cation exchange resin

resin was then washed with 8 liters of 1N HCl, followed by washing with 10 liters of deionized water until neutral.

A 1.9-liter aliquot of the total inorganic fraction (Sample 7) was passed through the Bio Rad AG 1-X8 anion exchange resin at a flowrate of 50 ml/min. The resin was then washed with 2 liters of deionized water until the eluate was clear. The 4 liters of dark brown eluate collected from the resin were concentrated to 600 ml on a hotplate. This was designated as sample 8. From fraction 8, 500 ml were set aside for bioassay testing and 100 ml were used for analysis.

2) anions

Prior to use, the Bio Rad 50W-X8 cation exchange resin (4 lb) was conditioned by washing with 10 liters of 1N HCl, 10 liters of deionized water and then 8 liters of 1N NaOH. A 1.65-liter aliquot of the inorganic fraction was passed through the resin column (6 cm ID x 125 cm height) at a flowrate of 50 ml/min. The column was washed with deionized water until the eluate was almost colorless. A total of 4.5 liters of eluate was collected and concentrated to 1.7 liters on a hotplate. During concentration, one-half of the sample was lost due to breakage of the beaker, leaving only 850 ml of the anion fraction. This was designated as sample 9; 650 ml were set aside as a bioassay test sample and 200 ml were used for chemical analysis.

e. separation of base/neutrals (sample 3)

A 1.5-liter aliquot of the total organic fraction was extracted three times with 300 ml methylene chloride at pH 14. The methylene chloride was dried over anhydrous sodium sulfate and concentrated on a steam bath to 4 ml using a K-D apparatus. The solution was then transferred with 10 ml DMSO to a round bottom flask and evaporated on a rotary evaporator at 50°C for 1 hour to remove all methylene chloride. Final volume of the base/neutral fraction was adjusted to 15 ml with DMSO and the fraction was retained for bioassay testing.

f. separation of acids (sample 4)

The remaining aqueous phase after the base/neutral extraction was acidified with 10N H_2SO_4 to a pH of 1 and was extracted three times with 300 ml of methylene chloride. The combined methylene chloride extracts were treated in the same manner as the base/neutral extracts described in the preceding paragraph. The final volume of the acid fraction (sample 4) in DMSO was adjusted to 20 ml and retained as a bioassay test sample.

g. separation of residuals (sample 5)

The residual aqueous phase remaining after the extraction of acids was concentrated to 1 liter. An 800 ml aliquot of the residual fraction (sample 5) was set aside for bioassay testing and a 200 ml aliquot was designated for subsequent chemical analysis.

3. Chemical analysis of collection A fractions

A Finnigan 4021 quadrupole mass spectrometer coupled to a 9610 gas chromatograph and an Incos 2300 data system were used to analyze the sample material. Compound separations were performed using a fused silica capillary column (30 m x 0.32 mm ID, 95 percent methyl, 5 percent phenyl silicone, 1 μm film thickness), splitless injection at 30°C followed by temperature programming to 300°C at 10°C/minute with injector temperature of 300°C, and helium carrier gas at 10 psi pressure. The mass spectrometer operating conditions included an ionizing voltage of 70 eV, 0.5 mA emission current, and was operated in a continuous monitoring mode which scanned from 50 to 500 amu at 450 amu per second.

Results from the analysis (computer assisted GC/MS) of whole waste material, the inorganic fraction, and the various organic subfractions indicate the presence of numerous organic compounds. Tables 13 through 17 provide a list of identified compounds, respective scan numbers (retention times), approximate compound concentrations, and matching library search indices (purity/fit indices). Since analyses were not also conducted using reference standards, all compound identifications should probably be regarded as tentative. The identification of positional isomers have been based on results of a National Bureau of Standards (NBS) library search without further verification of compound structure.

The purity index of compounds identified by the NBS library search and the availability of mass spectra were the major limiting factors encountered during the compound identifications. Whenever a compound identification was made through the library search, the mass spectrum was also manually examined. In many instances, the analysis allowed for the identification of isomers and for the assignment of coeluting peaks. Mass spectra with fit indices greater than 650 were recorded. However, if the respective sample

TABLE 13. SEMIVOLATILE ORGANIC COMPOUNDS IDENTIFIED IN THE ORIGINAL COLLECTION A WASTE SITE MATERIAL

Scan Number	Compound	Estimated Concentration (µg/l)	Matching Indices (purity/fit)
555	benzaldehyde	2,000	911/952
605	1-ethoxybutane	3,000	474/705
638	trans-2-chlorocyclohexanol	9,000	704/842
696	methylcycloheptane	4,000	408/731
726	5-methyl-1,2-hexadiene	9,000	575/652
734	3-ethyl-1,4-hexadiene	8,000	631/751
850	benzoic acid	8,200	733/945
859, 881 891, 908	methylpropoxycyclohexene (4 isomers)	150,000	M
960	1,2-benzenedicarboxylic acid	360	462/838
1004	propionilic acid butylester	1,400	523/791
1018	4-hydroxybenzeneacetic acid	2,900	754/950
1024	phenoxyacetic acid	730	670/916
1049	cyclopentane carboxaldehyde	5,400	622/781
1257, 1265	dimethyl benzoic acid methylester (2 isomers)	4,200	M
1339	pentachlorophenol	20,000	692/914
1360	o-benzyloxybenzylalcohol	380,000	390/676
1424	p-phenylcarbanilic acid	28,000	342/745
1503	9,10-anthracene-dione	16,000	711/828

M -- manual interpretation

Note: Results include the respective scan numbers (retention times), approximate compound concentrations, and matching library search indices (purity/fit indices). Most of the identifications should be classified as tentative since compound reference standards were not used. Results indicating the presence of positional isomers were based on the NBS library search.

concentrations were high, compounds with purity indices of 300 to 400 and with fit indices as low as 640 were also reported. For a perfect match, both purity and fit indices must be 1,000 while the presence of low purity (<750) and high fit (>900) typically indicate the presence of a coeluting compound.

Analysis of the inorganic fraction for organic compounds (Table 14) revealed that the fractionation scheme was not particularly effective at separating the organic compounds from the inorganic fraction. The previously

TABLE 14. SEMIVOLATILE ORGANIC COMPOUNDS IDENTIFIED IN THE TOTAL INORGANICS FRACTION OF COLLECTION A WASTE SITE MATERIAL

Scan Number	Compound	Estimated Concentration (µg/l)	Matching Indices (purity/fit)
555	benzaldehyde	720	911/952
605	1-ethoxybutane	1,100	474/705
638	trans-2-chlorocyclohexanol	32,000	704/843
696	methylcycloheptane	1,400	408/731
726	5-methyl-1,2-hexadiene	3,200	575/652
734	3-ethyl-1,4-hexadiene	2,900	631/751
850	benzoic acid	2,900	733/945
859, 881 891, 908	methylpropoxycyclohexene (4 isomers)	5,300	M
960	1,2-benzenedicarboxylic acid	130	462/838
1004	propionilic acid butylester	500	523/791
1018	4-hydroxybenzeneacetic acid	1,000	754/950
1024	phenoxyacetic acid	260	670/916
1049	cyclopentane carboxaldehyde	2,000	622/781
1257, 1265	dimethyl benzoic acid methylester (2 isomers)	1,500	M
1339	pentachlorophenol	7,200	692/914
1360	o-benzyloxybenzylalcohol	140,000	390/676
1424	p-phenylcarbanilic acid	10,000	342/745
1503	9,10-anthracene-dione	5,700	711/828

M -- manual interpretation

Note: Results include the respective scan numbers (retention times), approximate compound concentrations, and matching library search indices (purity/fit indices). Most of the data should probably be classified as tentative since compound reference standards were not used. Results indicating the presence of positional isomers were based on the NBS library search.

discussed attempts to revise the fractionation scheme (i.e., use of XAD-4 and XAD-8 resin columns) have not completely corrected the breakthrough of organic compounds. The presence of some organics should probably be expected since no fractionation scheme is likely to achieve complete separation and recovery of all organic compounds following a single extraction. However, this situation does cause a complication when subsequent bioassays are conducted. Under these conditions of partial compound separation, a bioassay result for the total inorganics sample would actually be a test response not only to the total

TABLE 15. SEMIVOLATILE ORGANIC COMPOUNDS IDENTIFIED IN THE BASE/NEUTRAL FRACTION OF COLLECTION A WASTE SITE MATERIAL

Scan Number	Compound	Estimated Concentration (μg/ml DMSO)	Matching Indices (purity/fit)
418	1,3-cyclopentadieneone	380	217/785
546	benzaldehyde	70	798/840
600	3-methoxybutanol	360	342/881
603, 613	2-methoxy-1-methylethoxy-2-propanol (2 isomers)	370	904/946 870/947
645	4-methylphenol	60	798/840
803, 842	2-butoxyethanol	120	382/989
970, 975	4-phenyl-3-butene-2-one (2 isomers)	30	860/977
1186	fluorene	13	690/961
1282	nonylphenol	80	572/893
1292	benzoic acid-1-methylethyl ester	240	569/947
1301	1-acetyloxy-1,2-diphenyl ethanone	120	230/966
1310	N-acetylbenzamide	350	594/925
1313	1,3-dimethyl-3-butenyl-4-methoxybenzene	70	680/825
1320	fluorene-9-one	100	660/933
1352	phenanthrene	180	842/996
1360	anthracene	240	801/961
1371	pentachlorophenol	20	772/901
1388	phenanthridine	110	740/991
1394	carbazole	200	841/966
1485	biphenylene	110	238/816
1496	9,10-anthracene-dione	420	871/986
1555	fluoranthene	130	847/977
1567	9-phenanthrene carboxaldehydeoxime	70	157/999
1581	2-methyl-9,10-anthracene-dione	70	626/972
1592	pyrene	90	869/991

Note: Results include the respective scan numbers (retention times), approximate compound concentrations, and matching library search indices (purity/fit indices). Most of the data should probably be classified as tentative since compound reference standards were not used. Results indicating the presence of positional isomers were based on the NBS library search.

inorganics but also to many organic compounds that have apparently been eluted with the aqueous phase (figure 17).

TABLE 16. SEMIVOLATILE ORGANIC COMPOUNDS IDENTIFIED IN THE ORGANIC ACIDS FRACTION OF COLLECTION A WASTE SITE MATERIAL

Scan Number	Compound	Estimated Concentration (μg/ml DMSO)	Matching Indices (purity/fit)
545	benzaldehyde	12	951/975
570	phenol	14	969/995
594	1-methoxy-1-methylethoxy-2-propanol	17	929/959
635	benzenemethanol	11	878/961
673	3-methyl-1,4-pentanediol	7	568/893
675	4-methylphenol	7	822/862
695	1-cyclohexen-1-yl ethanone	2	841/965
795	benzoic acid	5	917/982
861	4-methoxy-2,2,6-trimethyl cyclohexanone	1	462/274
990	phenoxyacetic acid	19	694/926
1020	4-hydroxy-3-methoxy-benzaldehyde	8	854/982
1094	4-hydroxy-3-methoxy-phenylethanone	5	864/984
1160	2,3,4,6-tetrachlorophenol	22	629/999
1247	9-decen-1-ol	38	386/954
1330	pentachlorophenol	100	831/993
1346	phenanthrene	10	820/988
1357	anthracene	13	861/996
1387	carbazole	4	641/972
1415	9-anthracenone	2	276/882
1435	2-methylanthracene	4	469/950
1475	dibenzofuran	5	412/952
1488	9,10-anthracene-dione	16	824/969
1551	fluoranthene	8	876/965
1566	pentadecenoic acid	14	525/976
1588	pyrene	5	849/987

Note: Results include the respective scan numbers (retention times), approximate compound concentrations, and matching library search indices (purity/fit indices). Most of the data should probably be classified as tentative since compound reference standards were not used.

Inorganic analytical results are shown in Table 18. Results are shown for the original collection A material (filtered waste), the total inorganics fraction, and for the cation and anion subfractions. Analyses for 23 elements and 7 anions were conducted using inductively coupled argon plasma spectroscopy (ICAP). Fluoride, chloride, nitrate, phosphate, sulfate, and

TABLE 17. SEMIVOLATILE ORGANIC COMPOUNDS IDENTIFIED IN THE ORGANIC RESIDUALS FRACTION OF COLLECTION A WASTE SITE MATERIAL

Compound	Estimated Concentration (μg/ml)
benzaldehyde	0.5
methylcycloheptane	2.6
5-methyl-1,2-hexadiene	3.7
3-ethyl-1,4-hexadiene	5.2
benzoic acid	1.0
methylpropoxycyclohexene	2.0
1,2-benzenedicarboxylic acid	0.6
4-hydroxybenzeneacetic acid	0.4
o-benzyloxybenzylalcohol	9.6
p-phenylcarbanilic acid	3.9
9,10-anthracene-dione	4.2

Note: Most of the data should probably be classified as tentative since compound reference standards were not used.

nitrite determinations were made by ion chromatography using a Dionex Model 125 ion chromatograph equipped with an AS-3 anion column. Results for the original collection A sample material and for the total inorganics fraction indicate a quantitative recovery for all cations and anions present in the original waste sample.

4. Fractionation of collection B

As stated previously, hazardous waste material was collected from two different waste sites and these collections are being referred to as collection A and collection B. The sites were selected not only because they contained known toxic chemicals but also because of the diversity of chemical compounds present at each location. Aqueous, organic, and solid phases are frequently all present in individual waste collections, but for collection A and collection B, the majority of the sample was aqueous. Inorganics present in the collection B waste ranged in concentration from μg/g to mg/g levels.

TABLE 18. INORGANIC CONCENTRATIONS FOR ORIGINAL COLLECTION A WASTE MATERIAL, TOTAL INORGANICS FRACTION, CATION SUBFRACTION, AND ANION SUBFRACTION (All concentrations given as mg/l)

	Original Waste Material	Total Inorganics Fraction		Cation Subfraction	Anion Subfraction	
	(sample 1)	(sample 7)	% Recovery	(sample 8)	(sample 9)	% Recovery
Aluminum	420	130	80	140		27
Antimony	<15	<6	--	<1		--
Arsenic	<15	<6	--	<1		--
Barium	< 7	<3	--	9.0		--
Beryllium	<1.5	<0.3	--	<0.05		--
Cadmium	14	4.7	87	9.8		57
Calcium	200	52	68	62		25
Chromium	440	140	83	450		84
Cobalt	130	41	82	84		53
Copper	710	190	70	410		47
Iron	470	160	89	570		100
Lead	3	<1.2	--	7.9		216
Magnesium	690	200	75	280		33
Manganese	38	11	75	16		35
Molybdenum	21	7.8	97	<0.2		--
Nickel	130	50	100	140		88
Selenium	<15	<6	--	<1		--
Silver	<15	<6	--	<1		--
Strontium	<1.5	<0.6	--	1.0		--
Thallium	20	6.6	86	<1		--
Titanium	<1.5	<0.6	--	<0.1		--
Vanadium	<7	<3	--	<0.5		--
Zinc	48	17	92	23		39
Fluoride	710	360	132		290	109
Chloride	14,000	4,500	84		3,400	65
Nitrate	31,000	11,000	92		8,000	69
Phosphate	<200	<200	--		<200	--
Sulfate	77,000	16,000	54		10,000	35
Nitrite	41,000	13,000	82		9,300	61
Cyanide	800	320	104		140	47

Note: Original extract concentrations have been multiplied by factors that attempt to correct for volume adjustments made during the fractionation. However, these corrections which allow for an estimate of element recovery (first in the total inorganic fraction and then in the cation and anion subfractions) have also artifically produced some recovery values that exceed 100 percent.

Collection B also contained numerous semivolatile organic compounds that ranged in concentration from 100 to 2,000 ng/g. These semivolatiles included alkyl substituted pyridines, quinoline, various hydrogenated quinolines, polycyclic aromatic hydrocarbons (e.g., fluorene, phenanthrene, anthracene, pyrene, and their methyl substituted derivatives), as well as several phenols (e.g., phenol, 2,4-dimethylphenol, 4-chloro-m-cresol, and 4-nitrophenol). Volatile organic compounds were also present in the ng/g concentration range. The collection B volatile organics included benzene, toluene, ethylbenzene, xylenes, benzaldehyde, C_2-alkyl cyclohexane, trichloroethylene, and tetrachloroethylene.

a. sample preparation

As was the case with collection A waste, the collection B waste was filtered prior to beginning the method demonstration trial. Approximately 3 liters of the waste sample were filtered under nitrogen at a pressure of 40 psi through a 0.45 μm millipore filter. The filtrate is designated as sample 1. A 300 ml subsample of the filtered material was set aside for potential biological testing, a 650 ml portion was retained for chemical analysis, and 2 liters were used for the subsequent fractionation. This is a departure from the method protocol in that sample 1 should actually be the unfiltered waste material. The possibility of obtaining extracts of solid material prior to beginning the fractionation process is briefly mentioned, but this procedural step was not included in the current demonstration.

Figure 18 shows the fractionation scheme used for collection B. The procedure was the same for both collection A and collection B fractionations but Figure 18 differs from Figure 17 in that the organic recombination sample (sample 6) is not shown. The recombination sample was not prepared for either of the two waste collections. Figure 18 also indicates that the initial sample was considered to be the filtered hazardous waste. Resulting fraction and subfraction volumes are also shown in Figure 18 which can be used as a guide to the respective quantities of material involved when fractionating a hazardous waste sample. However, these Figure 18 volumes were taken from the collection B fractionation, and variations will obviously occur based on the physical and chemical characteristics of a given waste.

Table 19 presents approximate concentration factors for the fractions and subfractions of waste collection B. As stated above, these values are based on the composition of the filtered waste material (sample 1).

b. separation of inorganics (sample 7) and organics (sample 2)

A 2-liter portion of the filtered waste sample was passed through an XAD-4 resin column (6 cm ID x 105 cm height) at a flowrate of 10 ml/min. After all of the sample passed through the resin bed, the resin was washed with 3 liters of deionized water at a flowrate of 20 ml/min. The disappearance of color in the eluate was used as an indicator for the completeness of the removal of water soluble material from the column. The sample colored the upper end of the resin bed blue. The aqueous eluate from the XAD-4 resin was collected and was then passed through an XAD-8 resin column (6 cm ID x 37 cm height) at a flowrate of 10 ml/min. The XAD-8 column was additionally washed with 2 liters of deionized water at a flowrate of 20 ml/min. The eluate

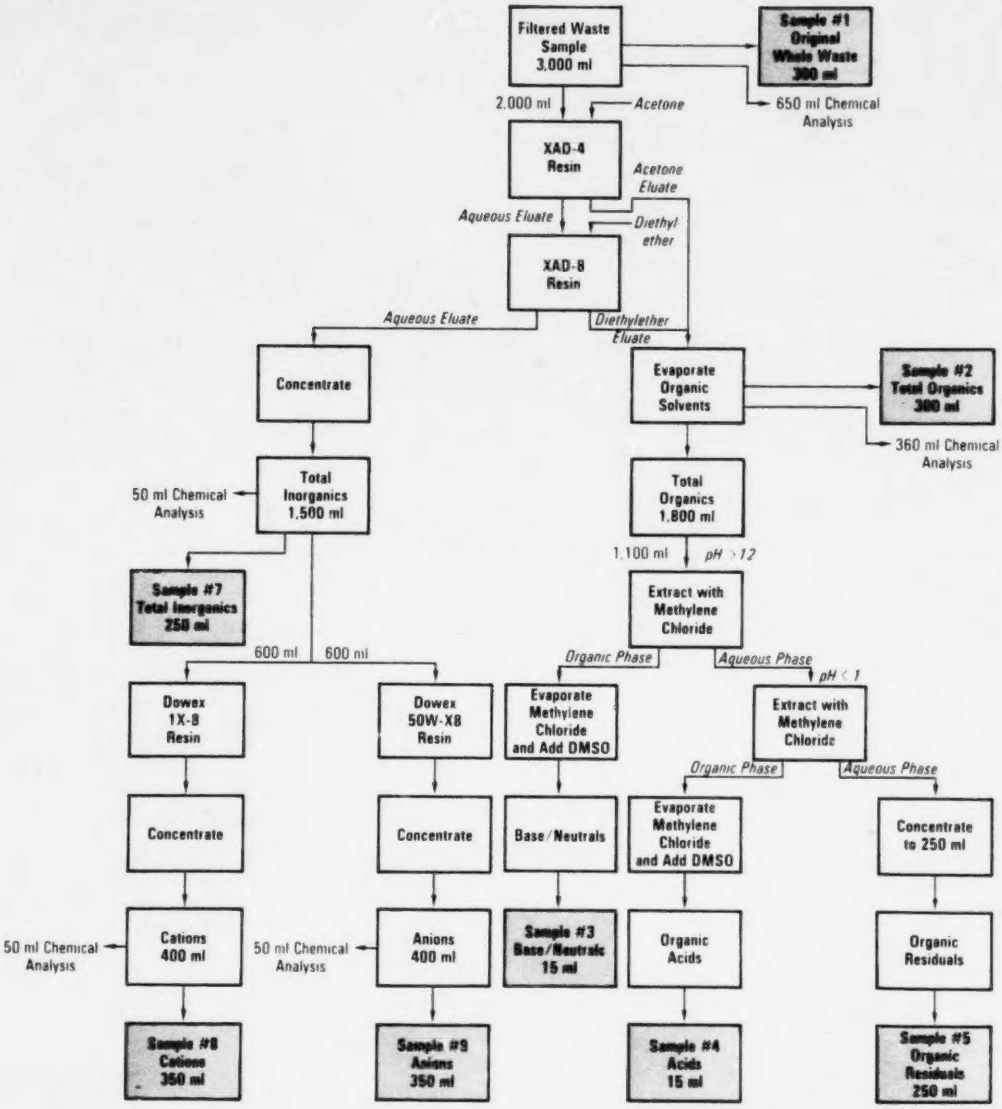
Filtered Waste Sample 3,000 ml
Sample #1 Original Whole Waste 300 ml
650 ml Chemical Analysis
2,000 ml
Acetone
XAD-4 Resin
Acetone Eluate
Aqueous Eluate
Diethyl-ether
XAD-8 Resin
Aqueous Eluate
Diethylether Eluate
Concentrate
Evaporate Organic Solvents
Sample #2 Total Organics 300 ml
360 ml Chemical Analysis
50 ml Chemical Analysis
Total Inorganics 1,500 ml
Total Organics 1,800 ml
1,100 ml
pH > 12
Sample #7 Total Inorganics 250 ml
Extract with Methylene Chloride
600 ml
600 ml
Organic Phase
Aqueous Phase
pH < 1
Dowex 1X-8 Resin
Dowex 50W-X8 Resin
Evaporate Methylene Chloride and Add DMSO
Extract with Methylene Chloride
Organic Phase
Aqueous Phase
Concentrate
Concentrate
Base/Neutrals
Evaporate Methylene Chloride and Add DMSO
Concentrate to 250 ml
50 ml Chemical Analysis
Cations 400 ml
50 ml Chemical Analysis
Anions 400 ml
Sample #3 Base/Neutrals 15 ml
Organic Acids
Organic Residuals
Sample #8 Cations 350 ml
Sample #9 Anions 350 ml
Sample #4 Acids 15 ml
Sample #5 Organic Residuals 250 ml

Figure 18. Fractionation scheme used to fractionate the collection B sample. Fraction and subfraction volumes are also indicated.

TABLE 19. FRACTION AND SUBFRACTION SAMPLE VOLUMES AND RELATIVE SAMPLE CONCENTRATION FACTORS RESULTING FROM CHEMICAL FRACTIONATION OF THE COLLECTION B WASTE SAMPLE

	Fraction Processing		Fraction Collection		Fraction Disposition			
Fraction[a]	Process Volume (ml)	Concentration Factor[b]	Collected Volume (ml)	Concentration Factor[b]	Fractionation Volume (ml)	Bioassay Testing Volume (ml)	Concentration Factor[b]	Analysis Volume (ml
1) Filtered waste sample (aqueous)	2,500	1.0	2,500	1.0	2,000	400	1.0	50
1) Filtered waste sample (aqueous)	3,000	1.00	2,000	1.0	2,000	300	1.0	650
2) Total organics (aqueous)	1,800	1.11	1,800	1.1	1,100	300	1.1	360
3) Base/neutrals (DMSO)	1,100	1.11	15	81.4	--	15	81.4	1
4) Acids (DMSO)	1,100	1.11	15	81.4	--	15	81.4	1
5) Residuals (aqueous)	1,100	1.11	250	4.9	--	250	4.9	10
7) Total inorganics (aqueous)	1,500	1.33	1,200	1.3	1,200	250	1.3	50
8) Cations (aqueous)	600	1.33	400	2.0	--	350	2.0	50
9) Anions (aqueous)	600	1.33	400	2.0	--	350	2.0	50

[a] For this demonstration trial, the original waste material was initially filtered as opposed to subsampling the unfiltered waste for use as sample 1. Relative concentration factors are therefore based on the filtered waste sample. The recombination of organic subfractions (sample 6) was not prepared during this phase of the demonstration trial.

[b] Concentration relative to filtered waste material.

and the washings were composited (7 liters) and were concentrated on a hotplate to 1.5 liters. The concentrate was designated as the total inorganics fraction (sample 7) and was then subsampled to include samples for potential bioassay testing (250 ml), chemical analysis (50 ml), and for further fractionation (1.2 liters).

c. separation of total organic fraction

The XAD-4 resin was eluted with 3 liters of acetone at a flowrate of 20 ml/min. The eluate began turning yellow after 1.5 liters of acetone were added but was clear after the 3 liters of acetone eluate were collected from the column. The XAD-8 resin was eluted with 1.5 liters of diethyl ether. Each eluate was concentrated separately on a rotary evaporator at 40°C and then combined to form a single fraction. This composite fraction (1.8 liters) was designated as total organics (sample 2). A 1.1 liter aliquot of this solution was used for base/neutral and acid extractions, 400 ml were used for GC/MS analysis, and 400 ml were set aside for biological testing.

d. separation of cations (sample 8) and anions (sample 9)

The total inorganic fraction (sample 7) was split into two equal aliquots. One aliquot was passed through a Bio Rad AG 1-X8 anion exchange resin to generate the cation sample. The other aliquot was passed through a Bio Rad 50W-X8 cation exchange resin to generate the anion sample. The amount of ion exchange resin required in each case was established from the conductivity measurements and from the data given by Pellizzari *et al.*, (1983). Table 21 shows the suggested and actual conductivity values *for the* cation and anion exchange columns.

TABLE 20. CONDUCTIVITY DATA AND RESIN VOLUMES FOR FRACTIONATION OF CATIONS AND ANIONS

	Conductivity (mhos/cm)	Resin Volume (ml)	Sample Volume (ml)	Resin to Sample Ratio
Suggested Values	3,000 to 6,000	10	5	2
Actual Value for Collection B Sample	1,200	3,600[a]	600[a]	6[a]
		2,260[b]	600[b]	3.76[b]

[a] Anion exchange resin
[b] Cation exchange resin

1) cations

A 600 ml portion of the total inorganic fraction was passed through a Bio Rad AG 1-X8 anion exchange column (6 cm ID x 125 cm height). The resin was washed with 2.5 liters of deionized water at a flowrate of 50 ml/min. Three liters of eluate were collected and concentrated on a hot plate to 400 ml. The concentrate was designated as sample 8. Two aliquots of 350 ml and 50 ml were separated for potential bioassay testing and for chemical analysis, respectively.

2) anions

Another 300 ml portion of the inorganic fraction was added to a cation exchange column (Bio Rad AG 50W-X8). The resin bed dimensions were 6 cm ID x 128 cm height. After the sample passed through, the resin was washed with deionized water (3 liters) at a flowrate of 50 ml/min. About 4 liters of eluate were collected and concentrated to 400 ml on a hot plate. The concentrate was designated as sample 9. A 350 ml portion of the material was retained as the designated sample for biological testing and the remainder (50 ml) was kept for potential chemical analysis. As discussed previously, when the chemical fractionation/aquatic bioassay test is routinely conducted, there are four component bioassays with several different sample dilutions required for each assay. It is, therefore, frequently necessary to conduct several replicate or repeat chemical fractionations to provide a sufficient quantity of material (i.e., volume/concentration) for biological testing. While replicate fractionation runs are being completed, those fractions and subfractions collected from the initial fractionations should be stored at 4°C prior to compositing the respective final eight samples. Neither the chemical analysis of sample material nor the biological assays should be conducted until the final fractionation samples are prepared.

e. separation of base/neutrals (sample 3)

Two 550 ml aliquots of the organic fraction were transferred to two 1-liter separatory funnels. The pH of the organic fraction was adjusted to approximately 14 with 1 to 2 ml of 10 N NaOH. Three extractions were then conducted using 100 ml of methylene chloride. The methylene chloride extracts were combined, dried over Na_2SO_4, and the solvent was removed in a Kuderna-Danish evaporator. After the solvent was concentrated to about 10 ml, DMSO was added (10 ml) and the evaporation was continued for about 30 minutes. The extract was then transferred to a roundbottom flask, the flask was rinsed with 1 to 2 ml of DMSO and the combined solution was evaporated for 30 minutes at 40°C on a rotary evaporator. The final volume was adjusted to 15 ml and the extract was designated as sample 3.

f. separation of acids (sample 4)

The remaining aqueous phase was acidified to a pH of approximately 1 with 2 to 3 ml of 10 N H_2SO_4. Three extractions were then conducted using 100 ml of methylene chloride. The sample 4 methylene chloride extract was prepared in the same way as was done for the base/neutrals subfraction. The final volume for the collection B organic acid subfraction (DMSO solvent) was 15 ml.

g. separation of residuals (sample 5)

The aqueous residue was concentrated to 250 ml on a hot plate. The concentrate was designated as sample 5 (organic residuals).

5. Chemical analysis of collection B fractions

Analytical results (computer assisted GC/MS) for collection B fractions are given in Tables 21 through 25. Analytical procedures were identical to those used for the collection A samples. Inorganic results for the analyses using inductively coupled argon plasma spectroscopy and ion chromatography techniques are given in Table 26. The approximate concentration factors have already been presented.

6. Summary of waste fractionation results

In the preceeding section, analytical results were given for the collection A and collection B samples. Computer programs (computer assisted GC/MS) confirmed the identification of many waste fraction and waste subfraction compounds, but the same compounds (those originally identified in the whole waste material) have not been followed through the fractionation procedure. Therefore, the recorded chromatograms and mass spectra were reviewed again and manual interpretations made in order to follow certain organic compounds through the entire fractionation.

Tables 27 and 28 show the recovery of selected compounds that were identified in the initial waste sample. Concentrations are given for the original sample material, the three organic subfractions, and for the total inorganics fraction. Obviously, the organics present in the inorganic fraction represent a deficiency in the procedure. Results are expressed in terms of the original 2-liter sample volume and have been derived by multiplying the respective extract concentration by those factors which correct for any volume adjustments and subsampling steps that occurred during the fractionation prior to obtaining the particular fraction or subfraction extract (i.e., the extract material that was chemically analyzed). Compound concentrations were determined by first identifying the compound through mass spectral interpretation, and then by comparing the total ion abundance of the respective compound with that of the nearest internal standard. The estimated detection limits provided in Tables 27 and 28 have been derived from results using internal standards and have attempted to include a consideration of the potential interferences that can result from the presence of numerous other compounds (i.e., adjacent spectral peaks).

Collection A contained fairly high concentrations of polar compounds and the overall recovery of these polar organics was usually not as efficient as the observed recovery of the base/neutrals. In addition, those polar compounds which were recovered from the XAD-4 and XAD-8 resins were frequently eluted with the aqueous phase into the total inorganic fraction. This situation will cause

TABLE 21. SEMIVOLATILE ORGANIC COMPOUNDS IDENTIFIED IN THE ORIGINAL COLLECTION B WASTE SITE MATERIAL

Scan Number	Compound	Estimated Concentration (µg/l)	Matching Indices (purity/fit)
281	pyridine	610	699/714
369	methylpyridine	660	753/782
454	2,6-dimethylpyridine	530	763/848
480	4-methyl-benzeneamine	240	831/897
555	1-ethenyl-2-pyridinone	400	736/888
589	C_3-alkylpyridine	410	M
609	phenol	550	M
618, 631, 635	C_3-alkylpyridine (3 isomers)	640	M
658, 669, 701, 712, 739	C_4-alkylpyridine (5 isomers)	1350	M
767, 772, 794, 845	C_5-alkylpyridine (4 isomers)	1200	M
825	1-methyl-1,2,3,4-tetrahydroisoquinoline	100	556/788
859	1,2,3,4-tetrahydroquinoline	630	691/891
862	tetrahydroquinoline isomer	280	M
876	quinoline or isoquinoline	350	M
893	5,6,7,8-tetrahydro-1-naphthaleneamine	140	485/733
897	C_3-alkylphenol	150	M
922	methyltetrahydroquinoline	640	M
972, 984	C_2-alkyltetrahydroquinoline (2 isomers)	710	M
1019, 1026, 1043, 1061, 1076	C_2-alkylquinoline (5 isomers)	710	M
1145	C_3-alkylquinoline	830	M
1162	C_3-alkylnaphthaleneamine	530	M
1190, 1232	C_5-alkylindoline	460	M
1309	C_5-alkylindol	100	M
1390	phenanthridine	130	734/937
1726	nonanedioic acid bis-(2-ethylhexyl) ester	300	553/694
1819	1,2-benzenedicarboxylic acid di-isooctyl ester	3200	767/873

M -- manual interpretation

Note: Results include the respective scan numbers (retention times), approximate compound concentrations, and matching library search indices (purity/fit indices). Most of the identifications should be classified as tentative since compound reference standards were not used. Results indicating the presence of positional isomers were based on the NBS library search.

TABLE 22. SEMIVOLATILE ORGANIC COMPOUNDS IDENTIFIED IN THE TOTAL INORGANIC FRACTION OF COLLECTION B WASTE SITE MATERIAL

Scan Number	Compound	Estimated Concentration (µg/l)	Matching Indices (purity/fit)
279	pyridine	400	709/738
367	methylpyridine	490	769/809
399	2-methyl-2-hexanol	150	636/745
452	2,6-dimethylpyridine	60	803/843

M -- manual interpretation

Note: Results include the respective scan numbers (retention times), approximate compound concentrations, and matching library search indices (purity/fit indices). Most of the data should probably be classified as tentative since compound reference standards were not used.

obvious data interpretation problems if the samples are subsequently tested using the component bioassays. Polar organics were also present in the collection B material, but they apparently did not overload the binding capacity of the resins and consequently were eluted into the total organic fraction.

The method demonstration using a complex sample material has therefore indicated some areas in which the fractionation procedure seems to be fairly effective, but it has also identified some areas where the protocol must be improved. In spite of the complex matrix of both waste site samples, the current fractionation procedure was reasonably effective at partitioning the neutral organics. The previously shown results for inorganic partitioning also revealed a reasonable efficient fractionation. However, it has also been noted that the partitioning of polar organics was not particularly impressive and that several of the alcohols and acids have been recovered in both inorganic and organic fractions. This breakthrough of polar organics was particularly dramatic when the original sample material contained high concentrations of these polar compounds (collection A). Instructions need to be added to the fractionation protocol that, when followed, will provide some indication of the appropriate sample volume to resin volume ratio required for each respective waste, waste leachate, or surface water sample. This ratio will not be the same for all samples as was noted with the collection A and collection B wastes. Perhaps a preliminary estimate of total organic carbon should routinely be made before each fractionation. Experiments using different resin columns and different solvents might also be needed before final protocol revisions are implemented.

Particulate matter present in the original waste material was ultimately filtered during the current fractionation to prevent problems from occurring with the resin columns. However, no attempt was made to remove any adsorbed

TABLE 23. SEMIVOLATILE ORGANIC COMPOUNDS IDENTIFIED IN THE BASE/NEUTRAL FRACTION OF COLLECTION B WASTE SITE MATERIAL

Scan Number	Compound	Estimated Concentration (μg/ml DMSO)	Matching Indices (purity/fit)
285	pyridine	17	792/830
324	methylpyridine	13	678/779
454	2,6-dimethylpyridine	3	M
515, 533, 574	C_2-alkylpyridine (3 isomers)	27	M
556, 592, 611, 619, 631, 634, 642	C_3-alkylpyridine (7 isomers)	66	M
658, 669, 682, 702, 711	C_4-alkylpyridine (4 isomers)	40	M
721, 725, 731, 749, 766, 773, 794, 850	C_5-alkylpyridine (8 isomers)	38	M
808	1,2,3,4-tetrahydroquinoline	7	734/927
878	quinoline	7	670/980
826, 834, 865, 895, 926, 930, 944	methyltetrahydroquinoline (7 isomers)	30	M
949	methylquinoline	15	611/968
982	C_2-alkyltetrahydroquinoline	8	M
999, 1017	C_3-alkyltetrahydroquinoline	22	
1052, 1062, 1079, 1099, 1114, 1130, 1149, 1164	C_2-alkylquinoline (8 isomers)	100	M
1176	2-ethyl-3,3-dimethylindole	16	472/958
1225	5,6,7,8-tetrahydro-2-naphthaleneamine	14	353/833 504/881
1233	3-ethyl-2,3-dimethylindoline	15	

M -- manual interpretation

Note: Results include the respective scan numbers (retention times), approximate compound concentrations, and matching library search indices (purity/fit indices). Most of the data should probably be classified as tentative since compound reference standards were not used. Results indicating the presence of positional isomers were based on the NBS library search.

TABLE 24. SEMIVOLATILE ORGANIC COMPOUNDS IDENTIFIED IN THE ORGANIC ACIDS FRACTION OF COLLECTION B WASTE SITE MATERIAL

Scan Number	Compound	Estimated Concentration (µg/ml DMSO)	Matching Indices (purity/fit)
410	2-methyl-2-hexanol	22.8	655/734
545	2-methyl-2,4-pentadiol	21.8	634/822
585	phenol	58.5	783/942
661	2-methylphenol	20.4	958/985
690	benzaldehyde	92.7	834/991
765, 788, 812	C_2-alkylphenol (3 isomers)	79.5	M
872, 900	C_3-alkylphenol (2 isomers)	22.4	M
931	3-hydroxybenzaldehyde	9.1	541/913
946, 966	C_2-alkylbenzaldehyde (2 isomers)	23.0	M
1015	C_4-alkylcyclohexanol	12.6	293/601
1093	2-methyl-3-propylcyclohexanol	5.1	678/842
1127	2,4,8,11,14-pentaoxapentadecane	7.8	732/829
1157	2,5,8,11-tetraoxadodecane	9.1	778/894
1393	1,1-biphenyl-2,2-diol	3.0	594/926
1455	1-methoxy-3-phenoxybenzene	2.1	479/912

M -- manual interpretation

Note: Results include the respective scan numbers (retention times), approximate compound concentrations, and matching library search indices (purity/fit indices). Most of the data should probably be classified as tentative since compound reference standards were not used. Results indicating the presence of positional isomers were based on the NBS library search.

compounds from the particulate matter, or to dissolve the particles prior to continuing the fractionation. Some specific sample treatment steps (not currently specified in the protocol) should be added that direct the laboratory to remove the sample particulate matter and to then add an extract of the particulate matter to the material being fractionated.

TABLE 25. SEMIVOLATILE ORGANIC COMPOUNDS IDENTIFIED IN THE ORGANIC RESIDUALS FRACTION OF COLLECTION B WASTE SITE MATERIAL

Scan Number	Compound	Estimated Concentration (μg/ml DMSO)	Matching Indices (purity/fit)
198	benzene	21.9	792/986
234	5,5-dimethyl-2-hexene	6.0	584/856
268, 305	C_2-alkylpentene (2 isomers)	32.6	M
423	3-methyl-2-butanoic acid methylester	7.0	715/883
564	1,3,5-trimethylbenzene	50.0	811/961
643	4,4-dimethyl-2,6-piperidinedione	30.4	509/753
731	2,6-dimethylheptadiene-4-one	59.8	790/908
741	1,2,3,4-tetramethylbenzene	44.0	769/989
755	3,5,5-trimethylcyclohexeneone	16.3	706/882
894	2,3-dimethyl-2-butenyl-benzene	18.7	813/982
910	2,4-dimethyl-1-methyldimethyl-benzene	2.4	579/924
1123	3,8-dimethylnaphthalenedione	16.6	551/875
1123	2,3,6-trimethylnaphthalene		461/953
1180	3,8-dimethylnaphthalenedione	45.6	510/860
1180	2,3,6-trimethylnaphthalene		503/965
1205	3,4-dihydronaphthaleneone	22.4	836/976
1223	1,2,3,4-tetrahydronaphthalene	8.9	674/959
1261	2-methoxy-1,1-biphenyl	5.2	677/924
1341	2,3-dihydroxydimethylindeneone	0.1	592/935
1410, 1418	dimethylpropylnaphthalenes (2 isomers)	12.9	802/981
1499	1,4-dimethoxynaphthalene	27.9	507/861

M -- manual interpretation

Note: Results include the respective scan numbers (retention times), approximate compound concentrations, and matching library search indices (purity/fit indices). Most of the data should probably be classified as tentative since compound reference standards were not used. Results indicating the presence of positional isomers were based on the NBS library search.

TABLE 26. INORGANIC CONCENTRATIONS FOR ORIGINAL COLLECTION B WASTE MATERIAL, TOTAL INORGANICS FRACTION, CATION SUBFRACITON, AND ANION SUBFRACTION (All concentrations given as mg/l.)

	Original Waste Material	Total Inorganics Fraction		Cation Subfraction	Anion Subfraction	
	(sample 1)	(sample 7)	% Recovery	(sample 8)	(sample 9)	% Recovery
Aluminum	110	160	190	160		73
Antimony	<2	<10	--	<1		--
Arsenic	<2	<10	--	<1		--
Barium	<1	<5	--	0.5		--
Beryllium	<0.1	<0.5	--	<0.06		--
Cadmium	<0.5	<2	--	0.26		--
Chromium	0.4	0.44	83	0.4		50
Cobalt	0.4	<2	--	0.88		110
Copper	<0.5	<2	--	0.64		--
Iron	1,300	1,800	104	1,900		73
Lead	0.7	<2	--	41		--
Magnesium	200	320	120	340		85
Manganese	30	43	108	48		80
Molybdenum	<0.5	<2	--	0.6		--
Nickel	1	2	150	2.4		120
Selenium	<2	<10	--	<1		--
Silver	<2	<10	--	<1		--
Strontium	0.5	2.4	360	1		100
Thallium	<2	<10	--	<1		--
Titanium	<0.2	<1	--	<0.1		--
Vanadium	<1	<5	--	<0.5		--
Zinc	15	51	255	38		127
Fluoride	100	30	23		9	4.5
Chloride	420	1,300	232		170	20
Nitrate	<40	<40	--		<20	--
Phosphate	<40	<60	--		33	--
Sulfate	4,400	20,000	340		5,900	67
Nitrite	<20	<30	--		<10	--
Cyanide	0.02	0.06	225		0.008	20

Note: Original extract concentrations have been multiplied by factors that attempt to correct for volume adjustments made during the fractionation. However, these corrections which allow for an estimate of element recovery (first in the total inorganic fraction and then in the cation and anion subfractions) have also artifically produced some recovery values that exceed 100 percent.

TABLE 27. RECOVERY AND PARTITIONING OF SELECTED ORGANIC COMPOUNDS FOLLOWING CHEMICAL FRACTIONATION OF COLLECTION A WASTE SITE MATERIAL

Compound	Whole Waste		Base/Neutrals		Organic Acids	
	Concentration^1 (µg/l)	Estimated D.L.^{2} (µg/l)	Concentration^1 (µg/l)	Estimated D.L.^{2} (µg/l)	Concentration^1 (µg/l)	Estimated D.L.^{2} (µg/l)
benzaldehyde	2,000	1000	740 (36)	50	170 (9)	20
benzoic acid	8,200	1000	ND	50	70 (1)	100
pentachlorophenol	20,000	1000	210 (1)	200	1,400 (7)	20
9,10-anthracenedione	16,000	1000	4,400 (26)	200	220 (1)	20
phenanthrene	ND	3000	1,900	200	140	20
anthracene	P	3000	2,500	200	180	20
carbazole	P	3000	2,100	200	50	20
biphenylene	ND	1000	1,200	50	70	20
fluoranthene	1,600	1000	1,400 (94)	50	110 (7)	20
pyrene	800	1000	950 (119)	50	70 (9)	20
p-phenylcarbanilic acid	28,000	1000	ND	50	ND	20
4-hydroxybenzene acetic acid	2,900	100	ND	50	ND	20
1,2-benzenedicarboxylic acid	360	100	ND	50	ND	20

1 - Calculated concentrations, given as µg/l, are based on a 2-liter total volume. The original extract concentrations have been multiplied by factors that correct for volume adjustments made during the fractionation. The percentage of compound recovered in a particular fraction is shown in parentheses.

2 - Estimated detection limit: Estimate derived from GC/MS analysis of reference samples and from the respective volume adjustments that occurred during the extraction process.

ND - Not detected

P - Present, but quantitative data not available due either to the presence of interfering compounds or to very low concentrations of the respective compound.

NA - Data not accessible

TABLE 27. (Continued)

	Organic Residuals		Total Inorganics		
Compound	Concentration [1] (µg/l)	Estimated D.L. [2] (µg/l)	Concentration[1] (µg/l)	Estimated D.L. [2] (µg/l)	Total Recovery (%)
benzaldehyde	350 (18)	NA	720 (34)	10	97
benzoic acid	700 (9)	NA	2,900 (36)	10	54
pentachlorophenol	ND	NA	7,200 (34)	10	42
9,10-anthracenedione	2,900 (18)	NA	5,700 (34)	10	79
phenanthrene	NA	NA	ND	10	
anthracene	NA	NA	ND	10	
carbazole	NA	NA	ND	10	
biphenylene	NA	NA	ND	10	
fluoranthene	NA	NA	ND	10	101
pyrene	NA	NA	ND	10	128
p-phenylcarbanilic acid	2,700 (10)	NA	10,000 (36)	10	46
4-hydroxybenzene acetic acid	280 (10)	NA	1,000 (34)	10	44
1,2-benzenedicarboxylic acid	420 (117)	NA	130 (36)	10	153

TABLE 28. RECOVERY AND PARTITIONING OF SELECTED ORGANIC COMPOUNDS FOLLOWING CHEMICAL FRACTIONATION OF COLLECTION B WASTE SITE MATERIAL

Compound	Whole Waste		Base/Neutrals		Organic Acids	
	Concentration[1] (μg/l)	Estimated D.L.[2] (μg/l)	Concentration[1] (μg/l)	Estimated D.L.[2] (μg/l)	Concentration[1] (μg/l)	Estimated D.L.[2] (μg/l)
pyridine	610	50	410 (67)	100	ND	100
2,6-dimethylpyridine	530	50	74 (14)	100	ND	100
phenol	550	20	ND	100	720 (131)	100
quinoline	350	20	172 (49)	100	ND	100
benzaldehyde	P	1,000	ND	100	1100	100
2-methyl-2-hexanol	ND	100	ND	100	280	100
methyl pyridine	660	50	320 (48)	100	ND	100
1,2,3,4-tetrahydro quinoline	630	50	172 (27)	100	ND	100

1 - Calculated concentrations, given as μg/l, are based on a 2-liter total volume. The original extract concentrations have been multiplied by factors that correct for volume adjustments made during the fractionation. The percentage of compound recovered in a particular fraction is shown in parentheses.

2 - Estimated detection limit: Estimate derived from GC/MS analysis of reference samples and from the respective volume adjustments that occurred during the extraction process.

ND - Not detected

P - Present, but quantitative data not available due either to the presence of interfering compounds or to very low concentrations of the respective compound.

TABLE 28. (Continued)

Compound	Organic Residuals		Total Inorganics		
	Concen-tration[1] (μg/l)	Estimated D.L.[2] (μg/l)	Concen-tration[1] (μg/l)	Estimated D.L.[2] (μg/l)	Total Recovery (%)
pyridine	P	10	300 (49)	200	116
2,6-dimethylpyridine	ND	10	45 (8)	200	22
phenol	P	100	ND	200	131
quinoline	ND	10	ND	200	49
benzaldehyde	ND	10	ND	200	
2-methyl-2-hexanol	ND	100	110	200	
methyl pyridine	ND	10	370 (56)	200	104
1,2,3,4-tetrahydro quinoline	ND	10	ND	200	27

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APPENDIX
METHOD PROTOCOL

CHEMICAL FRACTIONATION/AQUATIC BIOASSAY PROCEDURE: Application to Hazardous Waste Site Monitoring

February 1985

ENVIRONMENTAL MONITORING SYSTEMS LABORATORY
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I. Introduction

The chemical fractionation/aquatic bioassay technique can be used to identify bioactive fractions of a waste site collection, to assess some of the additive, synergistic and/or antagonistic effects caused by the component waste chemicals, and to provide a preliminary (or screening) hazard evaluation for the aquatic ecosystem. While chemical analysis of sample material is not a part of this fractionation/bioassay method, a combination of biological test data and chemical analytical data will allow for the identification of compounds and groups of compounds that present the greatest environmental hazard.

The overall procedure was initially developed by Walsh and Garnas at the U.S. Environmental Protection Agency's Environmental Research Laboratory in Gulf Breeze, Florida, and has been successfully used to test many chemically complex industrial effluents. The original Walsh and Garnas protocol was revised by the Environmental Monitoring Systems Laboratory in Las Vegas, Nevada, based on a series of extramural contract studies (Acurex Corporation and Bioassay Systems Corporation) in which actual hazardous waste site collections and synthetic waste (simulated leachate) were respectively used as sample material. Protocol revisions were necessary to address some of the problems frequently encountered with hazardous waste and to improve the completeness and clarity of all procedural instructions so that the technique could be considered for single laboratory testing (first step in the method validation process).

This procedure basically involves biological testing, first using a given sample of leachate, surface water, or liquid waste, and then using fractions and subfractions of the original sample material. The final test result is derived from a compilation of these different bioassay responses. Skeletonema costatum (saltwater algae), Selenastrum capricornutum (freshwater algae), Mysidopsis bahia (saltwater crustacean), and Daphnia magna (freshwater crustacean) are used as test organisms during the assay. However, the saltwater algae and saltwater crustacean tests are not conducted if an inland location of a particular waste site precludes any possibility of estuary contamination. There is a good data base available for these frequently used procedures that indicate assay responsiveness to many pollutant compounds, and some progress has been made toward method standardization. The test species are all of ecological importance to the respective freshwater and estuary environments; they are available commercially, and stock populations or cultures are fairly easy to maintain at the testing laboratory. The four component assays are screening procedures being used to test samples of unknown chemical composition, and for each of the respective assays, a minimum number of test organisms are used. The crustacean assays are both conducted under static conditions in which test solutions are not renewed during the test period. In spite of the preliminary test nature of these assays, the respective tests include eight different dilutions (concentrations) of the particular waste fraction being tested, a positive control, a negative control, and when applicable, a solvent control. Three replicates are routinely conducted for each of the eight sample dilutions and for each control.

The overall chemical fractionation/aquatic bioassay test result will serve as a basis for either a preliminary hazard prediction or as the basis for identifying those fractions or subfractions that should be chemically analyzed.

II. Initial Collection of Liquid Waste, Leachate, or Surface Water

A. Sample Types Prepared for Biological Testing

The chemical fractionation/aquatic bioassay procedure is used to test samples of leachate, surface water, or liquid waste in order to provide a preliminary (or screening) hazard evaluation for the aquatic environment. If a particular sample collection has been made from an inland location, and no reasonable possibility of estuary contamination exists, only two bioassay tests are used. If, by virtue of geographic location, estuary contamination is possible, four bioassay tests are ultimately used as part of the overall procedure. Before any biological testing is begun, dilution media must be prepared for the respective algal assays, and dilution water must be prepared for the crustacean tests.

It is assumed that the sample material has been collected from a waste site and has been brought to the laboratory for evaluation. A portion of this sample material is first tested for toxicity using the freshwater and marine algal bioassays and the freshwater and marine crustacean bioassays that compose the component parts of this overall test. If a toxic response (to include either stimulation or inhibition of the algal populations) does not occur in any of the component assays, no further testing is conducted using this particular waste collection. If a toxic response does occur for any of the component tests, a second portion of the waste material will then be chemically fractionated, and each fraction will be separately tested using the respective component bioassays.

Fractions and subfractions derived from the fractionation procedure include 1) original whole waste material, 2) total organics fraction, 3) base/neutrals subfraction, 4) organic acids subfraction, 5) organic residuals subfraction, 6) recombination of organic subfractions, 7) total inorganics fraction, 8) cation subfraction, and 9) anion fraction. When the complete evaluation is conducted, several liters of the original waste collection may be needed for chemical fractionation.

Data from the total test will be tabulated (9 sample types x 4 component tests = 36 separate test results for the original hazard waste collection). These results can then serve as the basis for either a preliminary hazard prediction or as the basis for identifying those fractions or subfractions that should be chemically analyzed.

B. Gross Estimate of Required Sample Volume

Table 1 presents some suggestions for the amount of sample material needed to conduct the component bioassay tests. Values shown are best estimates for waste samples of unknown chemical composition. As a gross estimate, between 40 and 80 liters of the hazardous waste material (i.e., liquid waste, waste leachate, or surface water) should probably be collected, but, in many instances, this amount would be excessive. The table is provided as an example only, but it does indicate the various sample types that must be available prior to starting the bioassay test.

TABLE 1. ESTIMATE OF SAMPLE MATERIAL REQUIRED TO TEST A SINGLE COLLECTION OF HAZARDOUS WASTE USING THE CHEMICAL FRACTIONATION/AQUATIC BIOASSAY[a,b] (liters)

Sample material	Component Bioassay Tests				Material Required for Subsequent Fractionation	Material Required for Subsequent Recombination	Total Quantity of Material Required
	Daphnia	Selenastrum	Mysidopsis	Skeletonema			
Whole waste material	2.0	0.5	4.0	0.5	30.1[c]	Not applicable	37.1
Total organics[d]	(2.0)	(0.5)	(4.0)	(0.5)	0.5[e]	Not applicable	f
Base/neutrals[d]	(2.0)	(0.5)	(4.0)	(0.5)	Not applicable	d,g	f
Acids[d]	(2.0)	(0.5)	(4.0)	(0.5)	Not applicable	d,g	f
Organics residuals[d]	(2.0)	(0.5)	(4.0)	(0.5)	Not applicable	d,g	f
Recombination[g]	(2.0)	(0.5)	(4.0)	(0.5)	Not applicable	Not applicable	
Total inorganics	2.0	0.5	4.0	0.5	18.0	Not applicable	25.0
Cations	2.0	0.5	4.0	0.5	Not applicable	Not applicable	7.0
Anions	2.0	0.5	4.0	0.5	Not applicable	Not applicable	7.0

[a] Quantities given represent waste and waste fractions, which in most cases would be subsequently diluted during the biological assay test phase. The entire table is somewhat hypothetical since the required quantities of test material will obviously depend on the material composition.

[b] Minimum volume estimate of 39.1 liters noted in Table 1 in Part a of the Protocol includes enough waste sample to conduct the fractionation/assay procedure and to chemically analyze the fraction samples.

[c] Represents volume that allows for chemical analysis.

[d] Quantities given represent reconstituted volumes of fractions or subfractions. Because most waste samples consist of a complex matrix of organics and inorganics, the organic and inorganic contents of each waste sample will differ. The quantity of media necessary to reconstitute organic fractions can be derived by use of the following calculation: the quantity of total sample that is fractionated divided by the total volume of the organic fraction or subfraction equals a concentration factor. This factor can then be used to estimate the volume of fraction or subfraction to be used.

[e] Represents one-half of the material extracted as total organics.

[f] Fractionating 30.1 liters as specified will probably produce enough sample material for all organic fractions and subfractions.

[g] Approximately 7 liters of hazardous waste material is probably required for the recombination sample. The equivalent of 2.3 liters of each reconstituted organic subfraction is prepared and added to a single container.

Several replicate or repeat chemical fractionations will probably be required to provide a sufficient quantity of total organics, total inorganics, base/neutrals, etc. It is not really feasible to fractionate the total required quantity of waste material in a single extraction/fractionation. The first batch of waste that is fractionated may, for example, result in 200 ml of base/neutrals when in fact 700 ml of this subfraction is the projected quantity required to complete the four component assays. The second, third, etc., fractionations should be completed and the resulting fractions composited with the respective fractions collected from previous runs. While replicate fractionation runs are being completed, those fractions and subfractions collected from the initial fractionation should be stored at 4°C prior to compositing the respective final eight samples. When the fractionation phase is complete, each of the eight samples should contain a sufficient volume (i.e., volume/concentration) to provide sample material for the four bioassay tests.

The collected waste material will probably be sent to the laboratory in a group of separate containers. Problems associated with sample collection and problems associated with achieving a representative sample are not a part of the assay method. For purposes of discussion, it is assumed that the contents of each respective container within a particular group are approximately equal in composition, i.e., subsamples of a single collection. While the orignial whole waste collection is assumed to be primarily a liquid, most samples collected from hazardous waste sites will probably include aqueous, organic, and solid phases. The initial bioassay tests are conducted by using the whole waste sample, but, if particulate matter is present, the larger particles should be removed with a sieve or screen. The remaining undissolved material should be dispersed (by gentle swirling of the container) before the material is used for testing. Another subsample(s) of the original whole waste will also be filtered if the initial bioassay shows a response and the overall test proceeds to the chemical fractionation stage.

Oxygen concentrations should be monitored during the biological test phase and these data should accompany the final test result. Particular attention should be noted for situations in which the dissolved oxygen concentration is less than 40 percent saturation in any of the test containers used during the Daphina assay and for concentrations less than 60 percent saturation during the Mysid test.

C. Overview of Sample Preparation Requirements

Samples for biological testing are basically prepared in one of two ways. Whole waste sample, total inorganic fraction, cation, and anion subfractions are prepared by adding directly to the sample those nutrients that are appropriate for the respective bioassay. Follow standard instructions for media preparation, but do not autoclave or otherwise sterilize the waste. This sample is then considered as the 100 percent concentration. Appropriate dilutions using culture media are mentioned in each of the four bioassay procedures. Nonaqueous fractions, including the total organic fraction, base/neutral subfraction, organic acid subfraction, residual subfraction, and the recombination sample are prepared by reconstituting an aliquot of the fraction concentrate as needed for each bioassay procedure. For example, if the total organic fraction had a volume of 900 ml, and a total of 30 liters of whole waste had been fractionated to produce this volume, the following ratio would exist between them:

$$\frac{\text{30 liters whole waste}}{\text{0.9 liter total organic fraction}} = \frac{33.333}{1}$$

If then, 2.0 liters of "reconstituted" total organics fraction was required for a particular bioassay procedure, the following ratios would be established to determine the amount of concentrate needed:

$$\frac{X}{\text{2.0 liters}} = \frac{1}{33.333}$$

$$X = 60.00 \text{ ml}$$

It would therefore require 60.00 ml of the total organics fraction to provide 2.0 liters of the 100 percent concentration. (Note: This is a theoretical 100 percent or original concentration because the sample material has not been chemically analyzed, and because the fractionation scheme will achieve neither complete compound separation nor total compound recovery.)

The sample is then prepared as the aqueous material: 60 ml total organics organics fraction in approximately 1 liter of deionized water. Appropriate nutrients are added in the amounts to supplement 2.0 liters. The total volume is adjusted to 2.0 liters with deionized water.

It is important to supplement the waste sample with the same nutrient concentrations found in the media to eliminate the possible effects of diluting out nutrients with unsupplemented waste. After a stock solution of nutrient-supplemented waste has been prepared (refer to specific bioassay procedures), dilutions can be made as directed in each bioassay procedure using the appropriate media. Volume estimates of nutrient-supplemented waste or reconstituted waste fractions that are required to provide enough material for dilution purposes are listed in Table 2.

With each set of instructions for the respective bioassays (presented in subsequent sections of this protocol), the testing laboratory is directed to prepare a series of concentrations or dilutions for each fraction i.e., 0.01, 0.1, 1.0, 10.0, 25, 50, 75, and 100 percent. However, this series of eight separate dilutions represents somewhat of a maxium testing situation, and the laboratory may elect to prepare fewer dilutions i.e., 0.01, 1.0, and 100 percent. If fewer dilutions are used, the total amount of each fraction or subfraction required will obviously be less.

III. Fractionation of Sample Material

A. Fractionation Objectives

This procedure is used to chemically fractionate the hazardous waste (or leachate) sample material into 1) total organics, 2) base/neutrals, 3)

TABLE 2. NUTRIENT-SUPPLEMENTED WASTE VOLUME ESTIMATES FOR INDIVIDUAL BIOASSAYS

Bioassay	Volume Estimates (liters)
Daphnia magna bioassay	2.0
Selenastrum capricornutum bioassy	0.5
Mysidopsis bahia bioassay	4.0
Skeletonema costatum bioassay	0.5

acids, 4) organic residuals, 5) recombination of 2, 3, and 4, 6) total inorganics, 7) cations, and 8) anions. The resulting fractions are used as sample material for the four component bioassay tests.

Chemical fractionation steps and resulting sample types for biological testing are shown in Figure 1. The sample is filtered through a 0.45 µm filter; the filtrate is passed through an XAD-4 resin to separate organic from inorganic compounds. The eluate from the XAD-4 resin is then passed through an XAD-8 resin. The organics are eluted from the XAD-4 and XAD-8 resins with acetone and diethylether, respectively. Following evaporation of the combined acetone and diethylether eluates, the remaining aqueous solution is extracted with methylene chloride at pH >12 to isolate the base/neutrals and at pH <1 to isolate the organic acids. The residual fraction is evaporated to dryness and dissolved in dimethylsulfoxide (DMSO) for bioassay testing. The inorganic fraction is further fractionated into anion and cation fractions using a Dowex 50W-X8 and Dowex 1-X8, respectively. Fractionation instructions are generally applicable to the processing of up to 2,000 ml of sample.

B. Sample Interferences

Method interferences can be caused by contaminants in the solvents, reagents, glassware, and other sample-processing hardware. All glassware should be cleaned with a nonionic detergent and hot water, and then rinsed thoroughly with tap water followed by deionized water. The cleaned glassware should then be air dried and heated in a muffle furnace at 400°C for 15 to 30 minutes. Solvent rinses with acetone and pesticide quality methylene chloride (distilled in glass) may be substituted for the muffle furnace heating. After drying and cooling, all glassware should be stored in a dust-free environment (either inverted or capped with aluminum foil). Use of high purity reagents will obviously help to minimize interferences. Purification of solvents by distillation in an all glass system might be done in some cases.

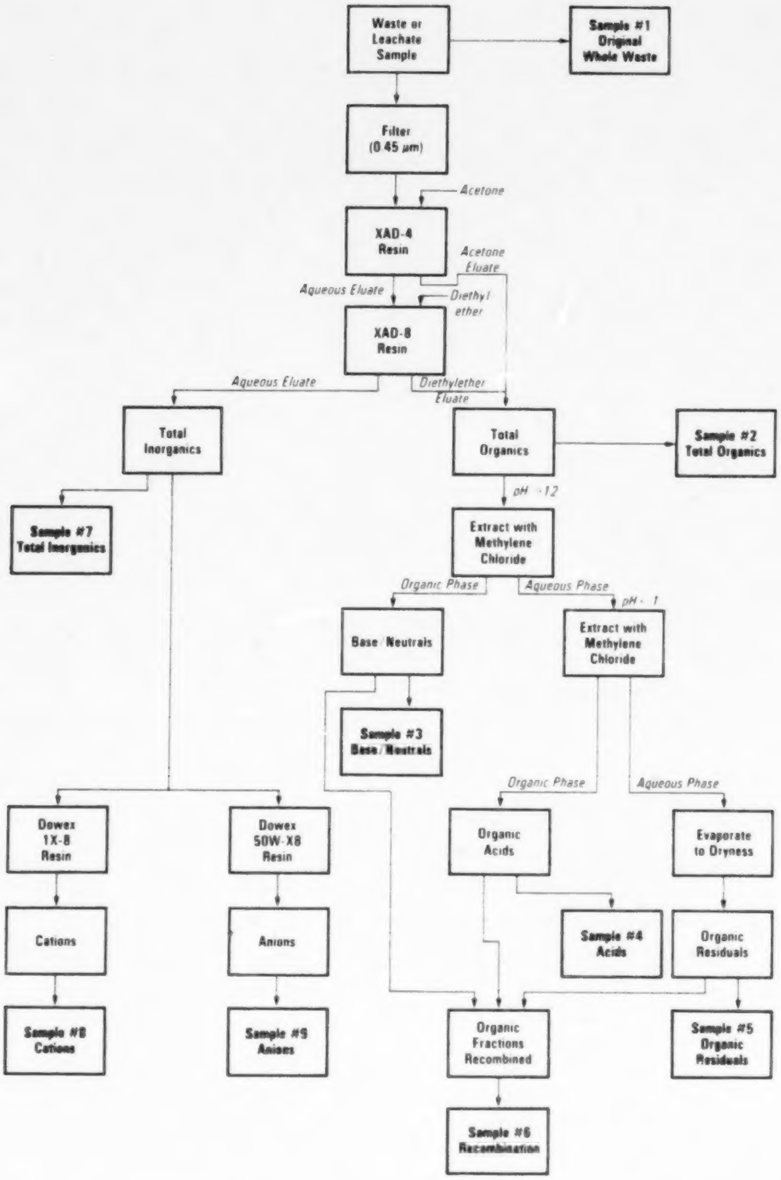
Waste or Leachate Sample
Sample #1 Original Whole Waste
Filter (0.45 μm)
Acetone
XAD-4 Resin
Acetone Eluate
Aqueous Eluate
Diethyl ether
XAD-8 Resin
Aqueous Eluate
Diethylether Eluate
Total Inorganics
Total Organics
Sample #2 Total Organics
Sample #7 Total Inorganics
pH >12
Extract with Methylene Chloride
Organic Phase
Aqueous Phase
pH < 1
Base/Neutrals
Extract with Methylene Chloride
Sample #3 Base/Neutrals
Organic Phase
Aqueous Phase
Dowex 1X-8 Resin
Dowex 50W-X8 Resin
Organic Acids
Evaporate to Dryness
Cations
Anions
Sample #4 Acids
Organic Residuals
Sample #8 Cations
Sample #9 Anions
Organic Fractions Recombined
Sample #5 Organic Residuals
Sample #6 Recombination

Figure 1. Chemical fractionation scheme and resulting sample material used during the chemical fractionation/aquatic bioassay procedure. For each hazardous waste collection, a total of nine separate samples will be biologically tested. These samples are 1) original whole waste material, 2) total organics, 3) base/neutrals, 4) acids, 5) organic residuals, 6) recombination of 3, 4, and 5, 7) total inorganics, 8) cations, and 9) anions.

C. Safety Considerations (Testing Laboratory should provide Safety Requirements)

The potential toxicity, carcinogenicity, etc., of the hazardous waste samples will not be known. Each sample should be treated as a potential health hazard. From this viewpoint, exposure to the chemicals present in the hazardous waste sample must be reduced to the lowest possible level by observing standard laboratory safety precautions for working with hazardous chemicals (i.e, wearing protective clothing, safety glasses, and gloves). Any information regarding the chemical composition of the hazardous waste sample should obviously be made available to the laboratory personnel.

D. Apparatus and Materials

Depending on the amount of material needed for testing and analysis, a suitably large quantity of the hazardous waste sample is chosen for fractionation. The amount of sample to be fractionated determines the size of the columns, the amount of resins, and the volumes of eluants and extraction solvents.

1. Materials

Filter apparatus for 142-mm filter

Glass fiber filter, 142-mm/0.45 μm

Nitrogen tank

Pressure gauge

Tubing

Soxhlet, 250-ml, 24/50 to 55/50 joints

Roundbottom flask, 1,000-ml, 24/40 joints

Condenser 55/50 joint

Pyrex beakers, 500 ml, 1,000 ml, 2,000 ml

Erlenmeyer flasks, 500 ml, 1,000 ml, 2,000 ml

Boiling chips

Graduated cylinders, 500 ml, 1,000 ml, 2,000 ml

Heated plate

Sample bottles, 500 ml, amber glass with Teflon-lined screw caps

Sample vials, 10 ml

Separatory funnels, 500 ml, with Telfon stopcocks

Chromatographic columns, 40 cm x 2 cm, with Teflon stopcock and 200-ml solvent reservoir

Kuderna-Danish (K-D) glassware:

- Synder columns, 3 bulb-macro
- Evaporation flasks, 500 ml
- Receiver ampules, 10 ml

Glass wool

Na_2SO_4 drying tubes

Steambath

Disposable pipettes

2. Reagents

Resins

- Amberlite XAD-4
- Amberlite XAD-8
- Dowex 1X-8, 20/50 mesh
- Dowex 50W X-8, 20/50 mesh

Methanol, analyzed for organic residue

Acetonitrile, analyzed HPLC grade

Diethylether, distilled in glass

Methylene chloride, distilled in glass

Acetone, analyzed for organic residue

Water, HPLC grade

Sodium hydroxide, 2N

Hydrochloric acid, 2N

Sodium sulfate, anhydrous

Sulfuric acid, 10N

Sodium hydroxide, 10N

pH paper

E. Fractionation Procedure

1. Sample preparation

The amount of each fraction required for biological testing will determine how much of the original sample is used for chemical fractionation. This procedure has been employed for fractionating up to 2,000 ml of hazardous waste. However, since 30 liters of the original waste or leachate may be needed, multiple or repeat chemical fractionations will frequently be required. The second, third, etc., fractionations should be completed and the resulting fractions composited with the respective fractions collected from previous runs. When the fractionation phase is complete, each of the eight fractions and subfractions should contain a sufficient volume (i.e., volume/concentration) to provide sample material for the four bioassay tests and for potential chemical analysis.

Mix sample well, prior to taking an aliquot for filtration. Filter through a 0.45-µm glass-fiber filter under nitrogen pressure (80 to 100 psi). When the pores of the filter become clogged (i.e., the flowrate of the filtrate becomes extremely slow), the filter needs to be exchanged. Nitrogen pressure is released slowly and the remaining sample left on the filter is drained into a container. Filtration is then continued following replacement of the filter. Waste samples will probably contain a solid phase which, in some cases, might be ground to smaller diameter particles prior to the filtration step.

2. Preparation of resins

a. XAD-resins

The XAD resin (XAD-4 or XAD-8, 50g, 16/15 mesh, Supelco number 2-0276 and 2-0278, or equivalent) is purified by three sequential continuous Soxhlet extractions using methanol, aceonitrile, and diethylether (each lasting approximately 8 hours). After the ether extraction, the solvent is decanted from the resin, methanol is added and the slurry is transferred to a glass bottle. Typical contaminants in the XAD-4 resins are naphthalene, ethyl benzene, and benzoic acid. Acetone dimers and isophorone have been observed when the XAD-4 resin is Soxhlet extracted with acetone. Therefore, Soxhlet extraction with acetone should not be used for purifying the XAD-4 resin.

b. ion-exchange resins

Dowex 50W-X8 (50g, 20/50 mesh, J. T. Baker or equivalent) is washed with 100 ml 2N HCl prior to loading it into a chromatographic column. The column is washed with deionized water until the eluate is neutral. 100 ml 2N NaOH is added to the column and the column is washed with deionized water until the eluate is neutral. This procedure removes impurities which may interfere with the ion exchange process and leaves the resin in Na^+ form ready for use. Typical contaminants are cations from the manufacturing process and low molecular weight organic impurities. Dowex 1X-8 (50g, 20/50 mesh, J. T. Baker or equivalent) is purified in the same manner, except that the column is first washed with 2N NaOH. Prior to use, 2N HCl is then passed through the column to exchange the resin in the Cl^- form.

3. Preparation of columns

The amount of waste sample to be fractionated determines column size and amount of resin to be used for sample fractionation. For example, to fractionate a 100-ml aqueous waste sample, a column with a 2-cm diameter, a height of 40-cm, and a 200-ml solvent reservoir is recommended. The column ends are fitted with Teflon stopcocks. Glasswool plugs are added to the bottom of the column as well as to the top of the resin bed. The resin-slurry is slowly poured into the column, such that after settling, the resin bed height is 40 cm. The column is washed with 1,000 ml of water before adding the sample (HPLC-grade water for the XAD resins and deionized water for the Dowex columns).

4. Sample fractionation

The fractionation of the waste sample is carried out according to the scheme given in Figure 1.

a. separation of organic and inorganic compounds

1) inorganics - An aliquot of the filtered hazardous waste sample is loaded on the XAD-4 column prepared as described above (Section 6.3). Organic freewater (HPLC water) is added immediately following complete transfer of the sample to the resin bed. The XAD-4 column is eluted with 1,000 ml HPLC water at a flowrate of approximately 10 ml/min. The aqueous eluate contains the inorganic compounds and any polar organic compounds that were not retained by the XAD-4 resin. This aqueous eluate is not passed through an XAD-8 resin. The aqueous eluate from the XAD-8 resin is labeled as the total inorganic fraction. Total volume of this fraction is first measured; then it is concentrated on a hot plate (40° to 50°C) to 300 ml for further fractionation. The inorganic fraction (300 ml) is split into three equal aliquots. One aliquot is labeled as total inorganics (Fraction 7). The other two aliquots are used for collection of cations (Fraction 8) and anions (Fraction 9).

Caution should be taken when concentrating the inorganic fraction. With certain types of waste samples it will not be possible to concentrate below a given volume because of high sample viscosity.

2) organics - The organic compounds are eluted from the XAD-4 column with 1,000 ml of acetone at a flowrate of 10 ml/min. The XAD-8 column is eluted with 1,000 ml of diethyl ether at a flowrate of 10 ml/min. The two eluates are composited and are concentrated to 150 ml using a K-D evaporator. The concentrate consists mostly of the water remaining in the XAD-4 and XAD-8 resins after the elution of inorganics. This concentrate is labeled as total organics (Fraction 2) and may be split as needed for bioassay testing, chemical analysis, and further fractionation. With certain types of hazardous wastes, Soxhlet extraction of the XAD-4/XAD-8 resins with methanol may be required following elution of the resins (acetone and diethyl ether) if color still persists after the elution.

b. separation of cations (sample 8) and anions (sample 9)

Two equal aliquots of the inorganic fraction, prepared as described above, are used for separation of cations and anions, respectively. One aliquot is transferred to a strong base anion exchange resin (Dowex 50W-X8) column; the other aliquot is transferred to a strong acid cation exchange resin (Dowex 1-X8) column. Each resin is eluted with 750 ml of deionized water. The eluate from the Dowex 50W-X8 resin contains the cations (Fraction 8); the eluate from the Dowex 1-X8 resin contains the anions (Fraction 9). Both fractions are concentrated to 100 ml on a hot plate at 40° to 50°C. Label the eluates as sample 8 for cations and sample 9 for anions.

c. separation of base/neutrals (sample 3) organic acids (sample 4), and organic residuals (sample 5).

1) preparation of drying column - Immediately prior to separating the base/neutral and acidic compounds, prepare two drying tubes for each extract. Place a small glasswool plug in the bottom of each column (5 cm x 20 cm) and add anhydrous sodium sulfate to a depth of 10 to 15 cm.

2) basic extraction - Transfer the aqueous concentrate (mentioned in item a, No. 2 above) to a separatory funnel. Basify to pH >11 with 2N NaOH. Add 100 ml of methylene chloride and extract. Repeat twice with fresh aliquots of methylene chloride. Combine the methylene chloride extracts and dry by passing the combined extract through a Na_2SO_4 column prepared as described above.

3) extract concentration - Add a boiling chip to the extract in the K-D evaporator and attach a three-ball Snyder column. Prewet the Snyder column by adding about 1 ml of methylene chloride to the column. Place the K-D apparatus on a steam bath (85°C) such that the concentrator tube is partially immersed in the hot water while the rounded surface of the flask is bathed with hot vapor. Adjust the position of the flask above the steam bath so that the balls of the Snyder column will actively clatter but the chambers will not flood with condensing solvent. When the extract volume is 8 to 10 ml, remove the K-D appartus from the steam bath and allow to cool for a few minutes. Add 10 to 50 ml DMSO and repeat the solvent concentration step. This solvent concentration step should remove over 95 percent of the methylene chloride. Dilute or concentrate as appropriate for bioassay test. Label as sample 3 (base/neutrals).

4) acidic extraction - The remaining aqueous phase from the basic extraction is acidified with 10N H_2SO_4 to pH <1 and extracted three times with 100 ml each of methylene chloride. The extracts are combined and concentrated to 8 to 10 ml using a K-D apparatus. Following the solvent exchange step described above (item C, No. 3), dilute or concentrate as appropriate for the bioassay test. Label as sample 4 (acids).

5) residual fraction (sample 5) - The aqueous phase remaining from the acidic extraction is evaporated to dryness on a hot plate at 40° to 50°C. The residue is dissolved in 10 to 50 ml of DMSO depending on the solubility of the residue. Label as sample 5 (residuals).

6. recombination sample (sample 6) - It should be remembered that aliquots of the base/neutral sample, the acid sample, and the organic residuals sample are recombined to provide another fraction (recombination sample) that will also be tested using the four bioassy procedures. Therefore, a sufficient quantity (volume/concentration) of the base/neutrals, acids, and residuals must be available, not only for bioassay testing and possible chemical analysis, but also to provide material for the recombination testing and associated potential chemical analysis.

F. Additional Control Samples

Before processing any samples, the laboratory should demonstrate through the fractionation of organic-free deionized water that the glassware and reagents do not give positive results to the bioassay test. Each time a set of samples is fractionated or there is a change in reagents, a laboratory reagent blank should be processed as a safeguard against laboratory contamination. It is also important to perform a control blank for the solvent exchange step since any traces of methylene chloride in the DMSO extract may produce a toxic response during the bioassays.

G. Storage of Samples Prior to Biological Testing

The hazardous waste samples and the resulting analytical fractions should be stored at 4°C. Thirty liters or more of the original waste collection may be required to complete the overall analysis (i.e., sufficient bioassay sample material from both the original waste collection and the various analytical fractions). It is not feasible to fractionate the total required quantity of waste material in a single fractionation. The second, third, etc. fractionations should be completed and the resulting fractions composited with the respective fractions collected from previous runs. While replicate fractionation runs are being completed, those fractions and subfractions collected from the initial fractionation should be stored at 4°C prior to compositing the respective final eight samples (samples that will then be used for biological testing).

H. Procedures Using Large Volumes of Liquid Waste, Leachate, or Surface Water

It can be assumed that large volumes of waste material will need to be fractionated in order to provide sufficient material for subsequent biological testing. The testing laboratory should determine the quantity of material to be used in a single fractionation run and whether multiple fractionations should be conducted simultaneously. It should again be emphasized that fractionation samples used during the bioassay tests are composite samples from the various fractionation runs and that the fractionation phase must be completed before any biological testing of fractionation samples is initiated.

Ratios between sample volume, amount of resin, volumes of solvent for elution and extraction, etc., should be kept basically the same as those presented in the fractionation instructions given above. The volume of hazardous waste that can be fractionated during a single run will obviously be limited by the size of the apparatus/glassware i.e., chromatographic columns, beakers, separatory funnels, etc. As the volume of material used in a given fractionation increases, larger volumes of solvent will be required to elute the resins which will then require larger collection vessels and longer elution times. Three chromatographic columns (3 cm ID x 60 cm height) with a combined volume of 1,270 cm^3 can be used to fractionate a 1-liter waste sample, but it will take almost twice as much time as it takes to fractionate 100 ml of the same waste material. In addition to the increased time for sample filtration, fraction collection, fraction concentration, etc., there is a greater possibility of compound degradation and/or evaporation since the fractions are kept for longer periods of time at room temperature (during column chromatography) and at temperatures up to 100°C (during concentration).

Quantitative recoveries can be obtained for acids, aldehydes, amines, esters, ethers, sulfur compounds, etc., when 4 to 7 ml of diethyl ether or when 6 to 10 ml of methanol are passed through a 0.5g XAD-4 resin spiked with 40μg of each test compound. However, acetone and diethyl ether elution efficiencies are not known for a wide variety of organic compounds, and the suggested volumes that are given in this protocol are best estimates based on previous results from hazardous waste fractionations.

As stated previously, when the respective bioassay tests are conducted using the various fractions, subfractions, and whole waste as sample material, the resulting data will allow the laboratory 1) to identify the bioactive waste fraction(s), 2) to assess some of the additive, synergistic, and/or antagonistic effects caused by the component waste chemicals, and 3) to prepare a preliminary (or screening) hazardous evaluation for the aquatic environment. Chemical analyses (e.g., GC/MS, ICAP) can also be conducted on those fractions and/or subfractions that have been shown to cause a toxic response. Although chemical anlaysis of sample material is not a part of this fractionation/bioassay procedure, a combination of biological test data and chemical analytical data will allow for the identification of compounds and groups of compounds that present the greatest environmental hazard. Portions of the fractions and subfractions obviously must be retained if chemical analyses are to be conducted.

IV. Aquatic Bioassays

A. Freshwater and Saltwater Algae

The freshwater green alga Selenastrum capricornutum and the saltwater diatom Skeletonema costatum are used as test species for the respective algal assays. Nutrient concentrations are typically less than those required to support maximal growth so that the test can respond to either inhibition or stimulation of algal growth. The freshwater and marine algae are obtained from commercial sources and are subsequently cultured using standard techniques. The algal assays are not begun until the respective cultures have demonstrated active growth, e.g., capable of logarithmic growth within the 96-hour test period. Eight nutrient supplemented samples (each containing a different concentration of the particular waste fraction or subfraction) and the respective control samples are each inoculated with 1 ml of 6- to 8-day old stock algal culture. Three replicates are conducted for each of the eight test samples and for each control. The flasks are incubated for 96 hours, at which time cell counts are performed using a hemacytometer and microscope to determine cell density. The percentage inhibition or stimulation of algal growth for each concentration, the EC_{10}, EC_{50}, and EC_{90}, and the sample dilution-response curve are determined from these counts. The final assay results are presented as either the EC_{50} (calculated concentration that will inhibit algal growth by 50 percent) or as the SC_{20} (calculated concentration that will stimulate algal growth by 20 percent). Either stimulation or inhibition could obviously be detrimental to a natural ecosystem.

1. Freshwater algae

a. basic test materials

1) test species - Selenastrum capricornutum 96-hour acute toxicity test (nonmotile freshwater green algae). The source of the organisms used in each assay will be specified with the resulting test data. Organisms used for each assay will be obtained from a single source. Cultures to be used in testing will be maintained at the test facility.

Age at start of study: 6- to 8-day-old stock of Selenastrum capricornutum. Each stock culture will be clearly identified with name of organism, date of subculture, and culture conditions.

2) materials

Analytical balance - capable of weighing 100 g with a precision of ±0.1 mg

Glass bottles - 100- to 500-ml capacity, sterile

Constant-temperature room of 24°C ± 2°C

Gyrotory shaker set at approximately 110 oscillations per minute (optional)

Continuous illumination of 450 ± 45 foot candles at the algal culture level provided by cool-white fluorescent bulbs

Light meter to monitor light intensity

Erlenmeyer flasks (125-ml), clean and sterile

Flask closures such as foam plugs or aluminum foil

Autoclave capable of 15 psi (1.1 kg/cm^2) and 121°C

Laminar flow hood

Microscope

Hemacytometer

Pipettes (sterile)

Algal nutrient media

An axenic culture of Selenastrum capricornutum

Reagents - See Tables 3 and 4

3) culture procedures

a) stock solutions: (may be stored at 2°C to 8°C in the dark for up to 6 months).

Macronutrients - 100-ml stock solutions of each of the salts at concentrations listed in Table 3 are prepared. Micronutrients - A single stock solution of 100 ml containing all micronutrient salts, except $FeCl_3$ and Na_2 EDTA $2H_2O$, at concentrations specified in Table 4 is prepared. $FeCl_3$-$Na_2EDTA \cdot 2H_2O$ - A single stock solution of 100 ml containing $FeCl_3$ and $Na_2EDTA \cdot 2H_2O$ at concentrations specified in Table 4 is prepared. All stock solutions must be autoclaved at 121°C (15 psi) for 20 minutes.

b) algal growth media: Algal growth media is prepared by adding 1 ml of each of the stock solutions in the order listed in Table 3 and 4 to 800-ml deionized water while stirring continuously. The pH of the media is adjusted to 7.5 by using 0.1- to 1.0-M solutions of HCl and NaOH. The final volume is brought to 1 liter with deionized water. The media is autoclaved as described above.

c) algal culture maintenance: Upon receipt of the culture, aseptically transfer a portion (usually 1.0 ml) to 40 ml of media in a 125-ml Erlenmeyer flask or 60 ml of media in a 250-ml Erlenmeyer flask. If an agar slant is received, add 1.0 ml of media to the slant and gently scrape the culture from the agar. Withdraw solution and organisms with pipette and transfer aseptically to growth media. The remaining cluture can be stored at 2° to 8°C (in the dark) for 6 months. Shake flasks at approximately 110 oscillations per minute under illumination of 450 ± 45 fc at 24°C or hand-shake flasks at least once per day. Subsequent stock transfers: Subculture algae weekly by aseptically transferring 1.0 ml of the week-old culture to new media. Use only 1- to 2-week-old cultures for testing purposes.

b. test procedure

Bioassay testing will begin using the original waste material. If a toxic response does occur (to include either stimulation or inhibition of the algal populations) for any of the component tests, a second portion of the waste material will then be chemically fractionated and each fraction will be separately tested using the four assays. Waste samples are tested for toxicity by establishing an EC_{50} (and SC_{20}) based on toxicity as a function of percentage waste in each test container.

TABLE 3. MACRONUTRIENT COMPOSITION OF FRESHWATER ALGAL ASSAY MEDIA

Compound	Concentration g/liter
$NaNO_3$	25.500
$NaHCO_3$	15.000
K_2HPO_4	1.044
$MgSO_4 \cdot 7H_2O$	14.700
$MgCl_2 \cdot 6H_2O$	12.164
$CaCl_2 \cdot 2H_2O$	4.410

TABLE 4. MICRONUTRIENT COMPOSITION OF FRESHWATER ALGAL ASSAY MEDIA

Compound	Concentration mg/ml
H_3BO_3	185.520
$MnCl_2 \cdot 4H_2O$	415.610
$ZnCl_2$	3.271
$CoCl_2 \cdot 6H_2O$	1.428
$CuCl_2 \cdot 2H_2O$	0.012
$Na_2MoO_4 \cdot 2H_2O$	7.260
$FeCl_3 \cdot 6H_2O$	160.000
$Na_2EDTA \cdot 2H_2O$	300.000

The leachate or waste sample is basically prepared in one of two ways: 1) Whole waste, total inorganic fraction, and cation and anion subfractions are prepared by adding nutrients directly to each. Enough sample must be prepared to allow for the appropriate number of dilutions and replicate containers. 2) Nonaqueous fractions, including total organics fraction, base/neutrals subfraction, organic acid subfraction, residual subfraction, and recombination of the organic subfractions are prepared by reconstituting the fraction or subfraction in the appropriate amount of water and then adding nutrients in the specified amounts indicated for media preparation.

During the fractionation procedure, organics become highly concentrated and are then resuspended in a known volume of organic solvent such as acetone or DMSO (dimethyl sulfoxide). If acetone is used to resuspend organic fractions, the concentration of acetone in each test container should not exceed 0.4 percent. If DMSO is used to resuspend fractions, the concentration of DMSO in each container should not exceed 1.0 percent.

When preparing waste material for the Selenastrum bioassay:

Place 400 ml of waste liquid or reconstituted waste fraction into a flask.

Add 0.5 ml of each stock solution listed in Tables 3 and 4.

Bring total volume to 500 ml by using waste liquid or reconstituted waste fraction, whichever is appropriate.

Adjust the pH of the solution to 6.5 to 7.5.

The above preparation is designated as the 100 percent concentration of the test material. Dilutions are prepared on a volume-percent basis (volume/volume) by mixing appropriate volumes of the test substance with algal media stock. When preparing dilutions of organic fractions or the recombination fraction, the culture media used should contain the same ratio of DMSO or acetone as that in the reconstituted organic preparation.

Eight concentrations of test material can be prepared along with positive and negative controls, all with three replicates. The concentrations (or dilutions) of sample material are 100, 75, 50, 25, 10, 1.0, 0.1, and 0.01 percent. However, fewer dilutions will frequently provide sufficient information. The test conditions are summarized in Table 5.

Volumes of media and waste or reconstituted waste are prepared as indicated in the ratios shown in Table 6.

A 6- to 8-day-old stock of Selenastrum capricornutum is used as the inoculum source. Calculate a volume of inoculum (0.1 to 1.0 ml) from the stock culture to yield an initial concentration of approximately 2×10^4 cells/ml. The volume of inoculum must remain constant for every test vessel. All experimental steps are carried out under sterile conditions.

TABLE 5. INCUBATION CONDITONS FOR FRESHWATER ALGAL ASSAY

Temperature	24°C ± 2°
Photoperiod, hours of light:dark	Continuous light
Light intensity	450 ± 45 fc
Container size (Erlenmeyer flask)	125 ml
Total volume of test container	40 ml (using gyrotory shaker) 25 ml (hand shaken)
Organisms per milliliter	2×10^4 cells/ml
Replicates	3
Test duration	96 hours
Shaking speed (oscillations per min. optional)	110 ± 10 opm

TABLE 6. RATIOS OF MEDIA TO WASTE (*Selenastrum capricornutum*)

Percentage Waste in Test Container[a]	Volume of Media, (ml)	Volume of Nutrient Supplemented Waste, (ml)
100	0	25
75	6.25	18.75
50	12.50	12.50
25	18.75	6.25
10	22.50	2.50
1.0	24.75	0.25
0.1	25.00	0.025
0.01	25.00	0.0025
0	25.00	0.0

[a] Indicates the concentration of whole waste or reconstituted waste fraction in each test container.

Incubation conditions for the freshwater algal assay are listed in Table 5. After 96 hours of incubation, algal growth is measured, by direct cell counting, with a hemacytometer (Neubauer) and a microscope. Two samples are taken from each flask and two counts are made of each sample. Record all cell counts (two counts per flask; six counts total per test concentration), and report the average count per concentration. Results should be recorded on Aquatic Bioassay Data Record Sheets - Selenastrum capricornutum (Figure 2). Fill both chambers of a Neubauer (1/10-mm depth) hemacytometer. Do not overfill the chambers. Cells are enumerated by counting cells in five of the nine large squares in each of the chambers. The total cell counts in each chamber are averaged. The average is then multiplied by 2.0×10^3 to arrive at a flask count in cells/ml.

c. tabulation of test result

The resulting percentage inhibition (I) or stimulation (S) of the algal population is calculated for each concentration.

$$\% I = (c-t)/c \times 100$$

$$\% S = (t-c)/c \times 100$$

c = mean growth (cell number) of the control

t = mean growth (cell number) of the treated culture.

For all samples, determine mathematically or graphically the EC_{50} and SC_{20}.

When the data are tabulated, the test result should include the derivation of stock cultures used during testing, the test conditions, and a mathematic and/or graphic interpretation of results expressed as EC_{50} and SC_{20}.

2. Saltwater algae

a. basic test materials

1) test species - Skeletonema costatum 96-hour acute toxicity test (marine algal). The source of the algae used in each assay will be specified along with test results. Algae used for each assay will be obtained from a single source. Age at start of study: Approximately 6- to 8-day-old cultures in log-phase growth. Each stock culture will be clearly identified with name of organism, date of subculture (culture initiation), and culture conditions.

2) materials

Analytical balance

Refractometer, salinity-conductivity meter, or salinometer. The instrument used must be checked monthly

DATA RECORD SHEET - *Selenastrum capricornutum* AQUATIC BIOASSAY

Test Sample:

Initiation: Date:________________ Time:________________
Termination: Date:________________ Time:________________

Waste %	Test container number	Cell counts (Well 1)	Cell counts (Well 2)	Average counts (1 and 2)	Average counts (3 flasks)	Cells per ml	%I	%S

Physical Parameters

Date	Temp (°C)	Light (fc)

Figure 2. Data Record Sheet - *Selenastrum capricornutum* aquatic bioassay.

for accuracy by checking the zero with deionized water and checking a mid- to high-range reading on the scale, i.e., 30 ppt salinity, with an appropriate salt standard solution. The solution may be prepared by the laboratory or it may be obtained commercially.

Autoclave capable of 15 psi at 121°C

Synthetic seawater mix (i.e., Rila^R Marine Mix)

Compounds listed in Table 7

Algal monoculture - liquid or agar slant

Erlenmeyer flasks - 125-ml, previously autoclaved for at least 30 minutes at 121°C and 15 to 25 psi

Flask stoppers - foam plugs or aluminum foil

Growth media

Shaker appartus - capable of 60 oscillations per minute (optional)

Constant temperature of 20°C ± 2° (daily measurements)

Photoperiod - 14 hours light/10 hours dark using cool-white fluorescent lighting at approximately 450 ± 45 foot-candles

Lugol's Iodine Fixative

3) culture procedures

The following procedures describe the preparation of nutrient media for the culture and maintenance of Skeletonema costatum. Marine Algal Assay Media (MAAM) is prepared in two parts. First, synthetic seawater is prepared, and second, nutrients are added to synthetic seawater from nutrient stocks (Table 7).

Synthetic seawater is prepared by dissolving a commercial sea salt (i.e., Rila Marine Mix) in glass-distilled water to a concentration of 30 parts per thousand. A 3.0-gallon aliquot is prepared in advance and stored in an acid-cleaned Nalgene polyethylene carboy.

Both test and stock media are prepared by using the same method and nutrient proportions. Approximately 800 ml of synthetic seawater is added to a 1-liter volumetric flask. Nutrients are added (30 ml of metal mix with EDTA, 2.0 ml of minor salt mix, and 1.0 ml of vitamin mix), and the flask is filled to the mark with seawater. Media is then aliquoted in appropriate amounts into culture or test flasks and autoclaved at 121°C (15 psi) for 20

TABLE 7. COMPOSITION OF MARINE ALGAL ASSAY MEDIA (MAAM) FOR 96-HOUR ACUTE TOXICITY TEST

Compound	Concentration	Amount Added to Stock Media
Metal mix		
$FeCl_3 \cdot 6H_2O$	0.048 g/liter	30.0 ml[a]
$MnCl_2 \cdot 4H_2O$	0.144 g/liter	
$ZnSO_4 \cdot 7H_2O$	0.045 g/liter	
$CuSO_4 \cdot 5H_2O$	0.157 mg/liter	
$CoCl_2 \cdot 6H_2O$	0.404 mg/liter	
H_3BO_3	1.140 g/liter	
Na_2EDTA	1.000 g/liter	
Minor salt mix		
K_3PO_4	3.0 g/liter	2.0 ml[a]
$NaNO_3$	50.0 g/liter	
$Na_2SiO_3 \cdot 9H_2O$	20.0 g/liter	
Vitamin mix		
Thiamine hydrochloride (in 95% Ethanol)	50.00 mg/100 ml	1.0 ml[a]
Biotin	0.01 mg/100 ml	
Vitamin B_{12}	0.10 mg/100 ml	

[a] Add indicated amounts of metal mix, minor salt mix, and vitamin mix to each liter of media.

minutes. Sterilized media must be used within 24 hours of preparation, but must be cool before use.

Upon receipt of culture, aseptically transfer a portion (usually 1.0 ml) to 50 ml of media in a 125-ml Erlenmeyer flask. If a slant is received, add approximately 1.0 ml of media and gently scrape the culture from of the agar. Transfer the solution to the growth media using a sterile pipette. Stock cultures of algae should be maintained at approximately 20°C ± 2° and a photoperiod of 14 hours light/10 hours dark in marine algal assay media. Cultures must be maintained on agar under axenic conditions. Transfers of stock cultures will be made approximately every 6 to 8 days to provide cultures with a sufficient number of cells growing exponentially for test inoculations.

b. test procedure

Bioassay testing will begin using the original waste material. If a toxic response does occur (to include either stimulation or inhibition of the algal populations) for any of the component tests, a second portion of the waste material will then be chemically fractionated and each fraction will be separately tested using the four assays. Waste samples are tested for toxicity by establishing an EC_{50} (and SC_{20}) based on toxicity as a function of the amount of waste (percentage) present in each test container.

The waste sample is basically prepared in one of two ways: 1) Whole waste, total inorganic fraction, and cation and anion subfractions are prepared by adding nutrients directly to each. Enough sample must be prepared to allow for the appropriate number of dilutions and replicate containers. 2) Nonaqueous fractions, including total organics fraction, base/neutrals subfraction, organic acid subfraction, residual subfraction, and recombination of the organic subfractions are prepared by reconstituting the fraction or subfraction in the appropriate amount of water and then adding nutrients in the specified amounts indicated for media preparation.

During the fractionation procedure, organics become highly concentrated and are then resuspended in a known volume of organic solvent such as acetone or DMSO (dimethyl sulfoxide). If acetone is used to resuspend the organic fractions, the concentration of acetone in each test container should not exceed 0.4 percent. If DMSO is used to resuspend fractions, the concentration of DMSO in each test container should not exceed 1.0 percent.

When preparing waste material for the _Skeletonema_ bioassay:

Place 500 ml of waste liquid or reconstituted waste fraction in a flask and add commercial sea salt mix to a concentration of 30 parts per thousand.

Take approximately 400 ml of this solution and add nutrients (15 ml of metal mix, 1.0 ml minor salt mix, 0.5 ml vitamin mix).

Bring to a final volume of 500 ml.

The above preparation is used as the 100 percent concentration of the test material. Dilutions are prepared on a volume-percent basis (volume/volume) by mixing appropriate volumes of test substance with algal media stock. When preparing dilutions of organic fractions or the recombination fraction, the culture media used must contain the same ratio of DMSO or acetone as that in the reconstituted organic fraction preparation.

Eight concentrations of test material can be prepared as well as positive and negative controls, all with three replicates. The concentrations (or dilutions) of sample material are 100, 75, 50, 25, 10, 1.0, 0.1, and 0.01 percent. The test conditions are summarized in Table 8.

TABLE 8. INCUBATION CONDITIONS FOR MARINE ALGAL ASSAY

Species	*Skeletonema costatum*
Temperature	20°C ± 2°
Photoperiod, hours of light:dark	14:10
Light intensity	450 ± 45 fc
Container size	125-ml Erlenmeyer flask
Total volume	40 ml (gyrotory shaker) 25 ml (hand-shaken)
Organisms per milliliter	8.0×10^4
Replicates	3
Test duration	96 hours
Shaking speed (oscillations per min., optional but recommended)	60 ± 10

Volumes of media and waste or reconstituted waste are prepared as indicated in the ratios shown in Table 9.

Media is prepared as described above and 50-ml aliquots are introduced into each 125-ml flask. The flasks are covered with foam stoppers and autoclaved at 15 psi and 120°C for 20 minutes. Transfers are accomplished by using a 6- to 8-day-old (approximately) stock. The flask is flamed and the transfer is made with a sterile pipette. The newly inoculated stock is placed in an incubator.

TABLE 9. RATIOS OF MEDIA TO WASTE (Skeletonema costatum)

Percentage Waste in Test Container[a]	Volume of Media (ml)	Volume of Nutrient Supplemented Waste (ml)
100	0	25
75	6.25	18.75
50	12.50	12.50
25	18.75	6.25
10	22.50	2.50
1.0	24.75	0.25
0.1	25.00	0.025
0.01	25.00	0.0025
0	25.00	0.0

[a] Indicates the concentration of whole waste or reconstituted waste fraction in each test container.

A culture of Skeletonema costatum (approximately 6 to 8 days old) is selected from the stock cabinet, and a sample is counted on a hemacytometer to determine the cell density. The amount of stock to be introduced to achieve a final concentration of 8.0 x 10^4 cells/ml is calculated (do not use greater than a 1.0-ml inoculum size). The inoculation is accomplished by using an automatic pipette with autoclaved plastic pipette tips. Each flask receives the same volume of stock culture. The flasks are arranged randomly in the test chamber and incubated for 96 hours. Hand-shaken flasks are shaken twice daily. Light and temperature are recorded daily. Incubation conditions for marine algal assays are given in Table 8. At 96 hours each flask receives 25 µl of Lugol's Iodine Fixative. The flasks are then covered with parafilm and stored in the dark at 2° to 8°C until they can be counted. No flask should be stored over 1 week. Algal growth is measured by cell enumeration with a hemacytometer (see counting instructions below). Results and physical parameter monitoring should be recorded on Data Record Sheet - Skeletonema costatum Aquatic Bioassay (Figure 3). Samples should not be diluted.

Fill both chambers of a Neubauer (1/10-mm depth) hemacytometer. Do not overfill the chambers. Cells are enumerated by counting cells in five of the nine large squares in each of the chambers. The total cell counts in each chamber are averaged, and the average is multiplied by 2.0 x 10^3 to arrive at the flask count in cells/ml.

c. tabulation of test result

Percentage inhibition (I) or stimulation (S) is calculated for each concentration:

$$\% I = {}^{(c-t)}/_{c} \times 100$$

DATA RECORD SHEET - *Skeletonema costatum* AQUATIC BIOASSAY

Test Sample:

Initiation: Date:__________ Time:__________
Termination: Date:__________ Time:__________

Waste %	Test container number	Cell counts (Well 1)	Cell counts (Well 2)	Average counts (1 and 2)	Average counts (3 flasks)	Cells per ml	%I	%S

Physical Parameters

Date	Temp (°C)	Light (fc)

Figure 3. Data Record Sheet - *Skeletonema costatum* aquatic bioassay.

$$\% S = {}^{(t-c)}/c \times 100$$

c = mean growth (cell number) of the control

t = mean growth (cell number) of the treated culture.

For all samples, determine mathematically or graphically the EC_{50} and SC_{20}.

When the data are tabulated, the test result should include the derivation of stock cultures used during testing, the test conditions, and a mathematic and/or graphic interpretation of results expressed as EC_{50} and SC_{20}.

B. Freshwater and Saltwater Crustaceans

These bioassays are conducted as static tests. The static test obviously requires less sample material than the flowthrough procedure which is sometimes a critical factor when planning assays for a limited amount of waste fractions and subfractions.

Mysid shrimp (Mysidopsis bahia) are used as the saltwater crustacean test organism and a brood culture is maintaned at the testing laboratory. Test conditions include the use of artificial seawater (i.e., marine salt mix and deionized water), eight dilutions of the particular waste fraction, and the required controls and replicates mentioned previously. At least five young adult mysids (5-6 days old) are used per test concentration and per control. The mysids are typically fed brine shrimp (Artemia sp.) during the 96-hour test. The assay result is expressed as an EC_{50} (calculated concentration or dilution that is lethal to half of the test organisms during the 96-hour period).

Eight concentrations of the particular waste fraction or subfraction are also tested using Daphnia magna (freshwater crustacean). The Daphnia assay is a 48-hour static test and, as with the other assays, three replicates are conducted for each dilution and for each of the control samples. Test solutions are kept on 250-ml beakers and are aerated during the test period. Dahnids less than 72 hours old are transferred to the test solutions by pipette and must have been thoroughly acclimated to the test conditions. Five daphnids are used for each test concentration and for each control. The number of immobilized or dead animals is recorded for each beaker at 24 and at 48 hours. The assay result is expressed as an EC_{50} (calculated concentration or dilution that is lethal to half of the test organisms during the 48-hour period).

1. Freshwater crustacean

a. basic test materials

1) test species

Daphnia magna 48-hour acute toxicity test (freshwater invertebrate-crustacean). The source of the organisms used in each assay must be specified with the assay result. Organisms used for each assay will be obtained from a single source. A culture history, including species, age, source, and growth media will be supplied with the test result.

Age at Start of Study: Early instars, less than 3 days old (72 hours). Number: 5 organisms per vessel.

Each vessel will be properly identified with date organisms were placed in the vessel, species name, concentration (if applicable), and culture conditions. From the time the test organisms are first cultured or received, they must be shielded from disturbances, and overcrowding must be avoided.

2) materials

Potassium chloride (KCl), reagent grade

Magnesium sulfate, heptahydrate
($MgSO_4 \cdot 7H_2O$), reagent grade

Sodium bicarbonate ($NaHCO_3$), reagent grade

Calcium sulfate, dihydrate ($CaSO_4 \cdot 2H_2O$) reagent grade

Deionized or distilled water

20-liter polyethylene or Nalgene type carboy

50- and 100-ml polyethylene bottles

1-liter carboys

Disposable pipettes or 10 ml-glass pipettes

5 and 10 gallon aquaria for culturing *Daphnia*

250 ml soft glass jars or Pyrex-type beakers

Trout chow (see food preparation section)

Dried yeast

Dried alfalfa

Eggs (optional, for feeding *Daphnia*)

Laboratory glassware detergent

pH meter

Dissolved oxygen meter

Centigrade thermometer

Test kit for measuring hardness of culture media

Conductivity meter

Environmental growth room or chamber (defined as having the ability to contol temperature and light within prescribed limits).

3) culture procedures

After collection or transportation, Daphnia will be held in, and acclimated to, the dilution water for 24 hours prior to the initiation of a test. A group of animals will not be used for a test if ephippia are present (i.e., the thick, rough brood chamber capsules formed around developing fertilized eggs), if they are immobilized, or if more than 5 percent die within the 24 hour interval before the test is scheduled to begin.

If a group fails to meet these criteria, they should not be used. Daphnia may be fed until the test begins.

Reconstituted water is used with the culture media because 1) it is of known quality, 2) it allows maximum growth and reproduction, 3) it produces predictable results, and 4) it is easily prepared. Reconstituted hard water (hardness: 160 to 180 mg/liter $CaCO_3$) should be used for the D. magna assay.

Reconstituted water media:

Reagent	Concentration mg/liter
$NaHCO_3$	192.0
$CaSO_4 2H_2O$	120.0
$MgSO_4 \cdot 7H_2O$	120.0
KCl	8.0

Compounds should be dissolved in deionized water and the media vigorously aerated for at least 2 hours. The initial pH of the media is approximately 8.0, but it may increase by as much as 0.5 as the Daphnia population increases. Although Daphnia can survive over a wide pH range, the optimum range is 7.5 to 8.5.

To make up media as needed (depending on how many cultures the laboratory maintains and the size of these cultures, usually once a week for each set of five stock cultures), prepare stock solutions of KCl, $MgSO_4$, and $NaHCO_3$ as follows:

KCl Stock Solution (expiration date, 1 year):

Place 8 g of crystalline, reagent grade KCl, in a 1-liter volumetric flask. Bring the volume to 1 liter with distilled or deionized water. Store in a 1-liter polyethylene bottle.

$MgSO_4$ Stock Solution (expiration date, 1 year):

Place 120 g of reagent grade, $MgSO_4 \cdot 7H_2O$, in a 1-liter volumetric flask. Bring the volume to 1 liter with distilled or deionized water. Store in a 1-liter polyethylene bottle.

$NaHCO_3$ Stock Solution (expiration date, 1 year):

Place 96 g reagent grade $NaHCO_3$ powder in a 1-liter volumetric flask. Bring the volume to 1 liter with distilled or deionized water. Store in a 1-liter polyethylene bottle.

Reconstituted water for daphnia assay:

Place 18.92 liters of deionized or distilled water in a 20-liter carboy. Add 2.4 g of $CaSO_4 \cdot 2H_2O$ to 1 liter of deionized or distilled water in a 2-liter beaker, and stir until dissolved. Add this solution to the carboy and mix well. Add each of the following stock solutions to the carboy and mix well after each addition:

20 ml KCl stock solution,
20 ml $MgSO_4 \cdot 7H_2O$ stock solution, and
40 ml $NaHCO_3$ stock solution. Vigorously aerate the medium in the carboy for 2 hours.

The media in each stock culture vessel will be replaced at seven day intervals. Siphon the old media out, using 5/16-inch plastic tubing (covered with fine mesh netting around the open end). Retain approximately 1/10 (300 ml) of the original media containing the *Daphnia* population. Pour this (old) media containing the *Daphnia* into a temporary holding vessel. Clean culture vessel. Fill newly cleaned vessel with fresh media. Gently pour contents of temporary holding vessel (old media and *Daphnia*) into vessel containing fresh media.

Daphnia populations will be thinned weekly to about 30 adults per stock vessel to prevent overcrowding, which may cause a population crash or the production of males and/or ephippia. Populations must be thinned during media replacement. To transfer *Daphnia*, use a disposable, jumbo-bulb pipette or a modified 10 ml serum pipette.

Liquid, containing adult *Daphnia magna*, can be poured from one container to another. Because the very young *Daphnia* are much more susceptible to air entrapment (carapace), they must be transferred from one container to another by pipette. The tip of the pipette must be kept under the surface of the liquid when the *Daphnia* are released.

Food preparation and feeding are of considerable importance in *Daphnia* culturing. A suspension of a trout chow, alfalfa, and yeast can be easily prepared and it will provide adequate nutrition. Place 6.3 g of trout chow pellets, 2.6 g of dried yeast, and 0.5 g of dried alfalfa into a blender. *Note:* The trout chow should conform to Fish and Wildlife Service Specification GR7-30 and can be obtained through livestock feed stores. Dried yeast (e.g., Fleischmann's dried yeast) can be obtained from many grocery stores. Dried alfalfa can usually be obtained at health food stores. Add 500 ml of distilled or deionized water. Mix for approximately 5 minutes. Place in refrigerator and allow to settle for 1 hour. Decant the top 300 ml and save (discard remainder). Place 30- to 50-ml aliquots in small polyethylene bottles with screw caps and store at -20°C ± 5°C. Thaw portions as needed. (After thawing, keep in refrigerator for maximum of 1 week, and then discard).

Feed 1.5 ml of prepared food per 1000 ml of media three times per week. There may be some excess food in the media at this rate of feeding; however, if the media is aerated and replaced each week, it should not cause a problem. As an alternate approach, hard-boiled egg yolks can be added to culture water until cloudy. The culture vessel will clear within 3 days, at which time more hard-boiled yolks can be added.

Ambient laboratory temperatures should remain in the range of 18° to 22°C during testing. Temperature fluctuations within any 12-hour period should not exceed 3°C. Ambient light intensities (50 to 100 fc) will be provided for *Daphnia* cultures. A minimum of 8 hours of illumination will also be provided. Culture vessels of clear glass should be used because they allow easy observation of the *Daphnia*. Size of the culture vessels will be approximately 3 liters.

Daphnia magna cultures should be maintained with dissolved oxygen concentrations of not less then 40 percent of saturation. Gentle aeration will be provided by an aquarium air pump and air stone. Dissolved oxygen concentrations should be determined at the start of new cultures and after media replacement or supplement. A dissolved oxygen meter can be used to make these determinations. Calibration of dissolved oxygen meters consists of performing an electronics check on the instrument before each use.

A minimum criterion for acceptable dilution water is that healthy organisms will survive in it for the duration of acclimation and testing without showing signs of stress (i.e., discoloration or unusual behavior). Water in which daphnids will survive and reproduce satisfactorily is an acceptable dilution water. The water should be of constant quality and should probably be analyzed to determine that none of the components listed in

Table 10 exceed allowable concentrations. The dilution water is considered to be of constant quality if the monthly ranges of the hardness, alkalinity, and conductivity are within 10 percent of their respective means and if the monthly range of pH is less than 0.4.

Early instars of Daphnia magna are used. To provide Daphnia for testing, gravid females are placed in brood tank(s) 72 hours prior to the initiation of the test. Each female will provide approximately 5 to 10 young during the first 24 hours. After 24 hours, adult females should be removed.

TABLE 10. ALLOWABLE MAXIMUM CONCENTRATIONS OF VARIOUS SUBSTANCES IN DILUTION WATER

Pollutants	Maximum concentration
Suspended solids	20 mg/liter
Total organic carbon	10 mg/liter
Nonionized ammonia	20 mg/liter
Residual chlorine	3 μg/liter
Total organophosphorus pesticides	50 ng/liter
Total organochlorine pesticides plus PCB's	50 ng/liter

b. test procedures

Bioassay testing will begin using the original waste material. If a toxic response does occur (to include either stimulation or inhibition of the algal populations) for any of the component tests, a second portion of the waste material will then be chemically fractionated and each fraction will be separately tested using the four assays. Waste or leachate samples are tested for toxicity by establishing an EC_{50} based on toxicity as a function of percentage waste in each test container.

The waste (or leachate) sample is basically prepared in one of two ways: 1) Whole waste, total inorganic fraction, and cation and anion subfractions are prepared by adding nutrients directly to each. Enough sample must be prepared to allow for the appropriate number of dilutions and replicates. 2) Nonaqueous fractions, including total organics fraction, base/neutrals subfraction, organic acid subfraction, residual subfraction, and recombination of the organic subfractions are prepared by reconstituting the fraction or subfraction in the appropriate amount of water and then adding nutrients in the specified amounts (indicated for media preparation).

During the fractionation procedure, organics become highly concentrated and are then resuspended in a known volume of organic solvent, such as acetone or DMSO (dimethyl sulfoxide). If acetone is used to resuspend organic fractions, the concentration of acetone in each test container should never exceed 0.4 percent. If DMSO is used to resuspend fractions, the concentration of DMSO in each test container should not exceed 1.0 percent.

The following guide is suggested when preparing waste material for the Daphnia bioassay: Place 1.89 liters of aqueous waste or reconstituted organic waste fractions into a 2.0-liter volumetric flask. Add 0.24 g $CaSO_4 \cdot 2H_2O$. Add 2.0 m KCl stock solution, 2.0 ml $MgSO_4 \cdot 7H_2O$ stock solution, and 4.0 ml $NaHCO_3$ stock solution.

The above preparation is used as the 100 percent concentration of test material. Additional test concentrations are prepared on a volume-percent basis by mixing appropriate volumes of the 100 percent concentration with culture media. When preparing dilutions of the organic fractions or of the recombined fractions, the culture media used for the dilutions must contain the same amount of DMSO or acetone as that in the reconstituted organic fraction preparation. Each dilution will, therefore, contain equivalent amounts of organic solvent. For the daphnid test, there must be 200 ml of solution or control water in each 250-ml beaker. Test organisms should be placed in the test and control vessels not more than 1 hour after the test solutions are prepared. Test conditions are outlined in Table 11.

Test concentrations for the original sample material, fractions, and subfractions, respectively, are 0.01, 0.1, 1.0, 10, 25, 50, 75, and 100 percent. It is assumed that the original collection is a liquid, and, in the case of hazardous waste or hazardous waste leachate, that this liquid will most likely contain aqueous, organic, and solid phases. In some cases, the original material (test sample 1) cannot be tested as collected (100 percent) because of such sample characteristics as viscosity, pH, etc. Any pretreatment of the original material to allow for testing would obviously be recorded, and the sample actually used for the first series of biological tests (adjusted to some volume/concentration as the original collection) would be designated as the 100 percent concentration.

The various dilution (concentration) volumes of media and sample material (or reconstituted sample material) are shown in Table 12. Five organisms per replicate (three replicates per concentration) are used for the assay. The same number of organisms per replicate are used for the controls.

The Daphnia test is conducted at 20°C ± 2°C. A photoperiod of 16 hours light and 8 hours dark must be used. Daphnia will not be fed during exposure.

Each test requires a control consisting of the same dilution water, conditions, procedures, and organisms as used in the test concentrations. If any solvent other than water is present in the test concentration, a solvent control is also required. Positive controls are also used with each series of dilutions.

Note: If no toxicity is detected at any concentration, and the 100 percent dose was tested, no further testing is required. The test

TABLE 11. SUMMARY OF REQUIRED TEST CONDITIONS FOR *Daphnia magna* TEST

Species	*Daphnia magna*
Temperature, °C (use centigrade thermometer)	20 ± 2
Photoperiod, hours light:dark	16:8
Water quality, hardness mg/liter as $CaCO_3$	Approximately 160-180
Container size	250 ml
Test volume	200 ml
Organisms per container	5
Replicates	3
Feed	No
Test duration	48 hours
Dissolved oxygen and pH measurement (using appropriate meters and electrodes)	0, 48 hours

TABLE 12. RATIOS OF MEDIA TO WASTE (*Daphnia magna* TEST)

Percentage Waste in Test Container[a]	Volume of Media (ml)	Volume of Nutrient Supplemented Waste (ml)
100	0	200
75	50	150
50	100	100
25	150	50
10	180	20
1.0	198	2.0
0.1	199.80	0.2
0.01	199.98	0.02
0	200	0

[a] Indicates the concentration of whole waste or reconstituted waste fraction in each test container.

material may be reported as having no detectable toxicity. Test materials that kill or immobilize all or nearly all of the test organisms can be retested with a lower dose range.

Figure 4 shows an example laboratory data sheet for recording test results.

The following measurements are required for the high, medium, and low concentrations as well as one negative control. They should be recorded at the beginning of the bioassay and after 48 hours (completion of testing).

pH--measure in low, medium, and high dose levels.

Dissolved oxygen--measure in low, medium, and high concentrations.

Conductivity--measure in low, medium, and high concentrations.

Temperature--of at least one vessel.

Mortality is the effect most often used to define acute toxicity to aquatic organisms. Because death is not always easily determined with some invertebrates, and EC_{50} is calculated rather than an LC_{50}, the principal criterion for effect on Daphnia is immobilization (defined as lack of movement except for minor activity of appendages). Mortality or immobilzation and abnormal behavior should be recorded every 24 hours for the duration of the test. Dead or immobilized organisms must be removed as soon as they are observed. If more than 10 percent of test organisms in any control die or are immobilized, (other than the positive control), the entire test series should be repeated, if sufficient material is available.

c. tabulation of test result

An EC_{50} determination will be provided. Binomial, Moving Average, or Probit Analysis methods can be used to determine EC_{50} values. When the data tabulations are complete, the test result should include the derivation of stock cultures used during testing, the test conditions, and a mathematic and/or graphic interpretation of results expressed as EC_{50}.

2. Saltwater crustacean

a. basic test materials

1) test species

Mysidopsis bahia (mysid shrimp) 96-hour acute toxicity test (oppossum shrimp). The source of the organisms used in each assay will be specified with the test results. Organisms used for each assay are obtained from a single source. (Age at Start of Study: Young adult).

Data Record Sheet - *Daphnia magna* Aquatic Bioassay

Test Sample:

Initiation: Date ______________ Time ______________________

Termination: Date ______________ Time ______________________

Test Temperature Range:

Time	0 h	24 h	48 h
Temp. (°C)			

Waste %	Test container number	Number of live organisms		Dissolved oxygen, mg/liter		pH		Conductivity	
		0 h	48 h	0 h	48 h	0 h	48 h	0 h	48 h

Figure 4. Data Record Sheet - *Daphnia magna* aquatic bioassay.

Number: Five (triplicate containers of each concentration). Each lot will be given a number upon receipt from the supplier. The number will be recorded on a card or sticker and placed on each container. No population of shrimp will be used for testing in which more than 5 percent of the population have died during the 48 hour internal immediately prior to the beginning of the test.

2) materials

Artificial seawater mix (such as Rila^R Marine Mix)

Refractometer or salinometer

Aquaria (19 to 76 liters) equipped with undergravel filtration system and bed of crushed oyster shell

Centigrade thermometer

Environmental growth room or chamber (defined as having the ability to control temperature and light within prescribed limits)

Dissolved oxygen meter

pH meter

1 liter (soft glass) containers or 8-inch culture dishes

Brine shrimp eggs

Tetra-min^R fishfood flakes

Graduated cylinders (500 and 1000 ml)

3) culture procedures

Cultures should be maintained in reconstituted seawater which is prepared by adding an artificial marine salt mix, such as Rila^R Marine Mix, to deionized water (salinity, 32 to 36 ppt). Salinity will be determined weekly with a refractometer. The refractometer should be checked monthly using deionized water as a low reference and an appropriate salt solution of known specific gravity as a high reference sample. These control or reference samples can be obtained commercially or may be prepared by the laboratory. The culture media will be aged to allow buildup of bacteria in the substrate. The culture media (synthetic seawater) is added to an aquarium containing the shells and is conditioned (aged) for at least 2 weeks before the mysids are introduced. During this period the water is circulated through the undergravel filter. The tops of the two bubble-up standpipes (from the filters) are placed immediately above the water surface. The rate of water flow through the filters must be sufficient to establish a moderate current in the aquarium. This will be evident in the orientation of the shrimp, which commonly align themselves with the current.

If brood stocks used for testing are maintained at the test facility, 19- to 76-liter aquaria should be used. The aquaria must be equipped with undergravel filters in a recirculating system. The bottom should contain about 5 cm of crushed oyster shells. Crushed oyster shells can be obtained through most large pet supply dealers. Oyster shells should be autoclaved before use.

Temperature must be maintained within a range of 20°C to 26°C (+ 2°C of test temperature). If necessary, a glass aquarium heater can be used. Fourteen hours of 50 to 100 fc illumination must be provided each day. Frequent feeding of live food is necessary to prevent cannibalism of the young by the adults. Newly hatched brine shrimp (Artemia) will be fed to Mysids, supplemented by Tetra-min^R fishfood flakes.

During holding, acclimation, and testing, the organisms must not be disturbed unnecessarily, either by excessive handling or by excessive movement around the tanks. Handling must be done as gently, carefully, and quickly as possible. The mysid holding temperature is between 22°C and 25°C, with a salinity range of 32 to 36 ppt. The dissolved oxygen concentration of dilution water should be between 90 and 100 percent saturation. Temperatures must be recorded daily. Salinity and dissolved oxygen concentration must be checked weekly. Salinity is checked with a salinometer or with a refractometer. The instrument must be calibrated using a water and salt solution of known concentration. Instrument checks should be performed on a monthly basis. Dissolved oxygen meters should be calibrated daily. Electronic calibration checks should be performed according to manufacturer's specifications.

b. test procedures

Bioassay testing will begin using the original waste material. If a toxic response does occur (to include either stimulation or inhibition of the algal populations) for any of the component bioassay tests, a second portion of the waste material will then be chemically fractionated and each fraction will be separately tested using the four bioassays. As stated before, the saltwater algae and saltwater crustacean tests are not conducted if an inland location of a particular waste site precludes any possibility of estuary contamination.

Waste (or leachate) samples are tested for toxicity by establishing an EC_{50} based on toxicity as a function of percentage waste (dilution) in each test container.

The waste sample is basically prepared in one of two ways. 1) Whole waste, total inorganic fraction, and cation and anion subfractions are prepared by adding nutrients directly to each. Enough sample must be prepared to allow for the appropriate number of dilutions and replicate containers. 2) Nonaqueous fractions, including total organics fraction, base/

neutrals subfraction, organic acid subfraction, residual subfraction, and recombination of the organic subfractions are prepared by reconstituting the fraction or subfraction in the appropriate amount of water and then adding nutrients in the specified amounts indicated for media preparation.

During the fractionation procedure, organics become highly concentrated in a known volume of organic solvent such as acetone or DMSO (dimethyl sulfoxide). If acetone is used to resuspend organic fractions, the concentration of acetone in each test container should not exceed 0.4 percent. If DMSO is used to resuspend fractions, the concentration of DMSO in each test container should not exceed 1.0 percent.

When preparing waste material for the *Mysid* bioassay:

Place approximately 3.5 liters of the liquid waste or reconstituted organic waste fraction into a large (i.e., 6.0-liter) flask. Add marine salt mix as indicated by manufacturer to between 32 and 36 ppt. Recheck volume and add enough of the liquid waste or reconstituted organic waste fraction to bring the total volume to 4 liters.

The above preparation is used (and designated) as the 100 percent concentration of test material. Additional test concentrations are prepared on a volume-percent basis by mixing appropriate volumes of the 100 percent concentration with culture media. When preparing dilutions of the organic fractions or the recombined fractions, the culture media used for the dilutions should contain the same ratio of DMSO or acetone as that in the reconstituted organic fraction preparation.

Eight concentrations (or dilutions) are prepared for the original sample material and for each separate fraction and subfraction (if chemical fractionation of the sample is conducted). As discussed above, fractionations and subfractions are first diluted to the original prefractionation volume (reconstituted 100 percent concentration). Biological tests or chemical analysis of sample material could also be conducted on a more concentrated fraction or subfraction sample. However, the prefractionation volume is always the basis for the 100 percent concentration shown in Table 13.

Five mysid shrimp per replicate per concentration are exposed to the respective Table 13 concentrations for 96 hours. There are three replicates conducted for each concentration and for each control. Five shrimp per replicate are also used for the positive control, negative control, and solvent control (if applicable).

Tests are conducted at a temperature between 22°C and 25°C. Continuous light must be provided under ambient laboratory lighting conditions (approximately 50 to 100 fc). During testing, the mysids will be given approximately twenty 48-hour-old *Artemia nauplii* per mysid per day. Test vessels will be either 1-liter (soft-glass) containers or 8-inch culture dishes. Organisms are placed in the test and control vessels not more than 1 hour after the test solutions have been prepared and aliquoted.

Table 14 summarizes the test conditions.

TABLE 13. DILUTION OF SAMPLE MATERIAL (ORIGINAL SAMPLE, FRACTIONS, AND SUBFRACTIONS) INTO A SERIES OF EIGHT CONCENTRATIONS FOR BIOLOGICAL TESTING (*Mysidopsis bahia* Test)

Approximate Percentage of Material in Test Container[a]	Volume of Media (ml)	Volume of Nutrient Supplemented Waste (ml)
100	0	500
75	125	375
50	250	250
25	375	125
10	450	50
1.0	495	5.0
0.1	499.50	0.5
0.01	499.95	0.05
0	500	0

[a] Indicates the concentration of whole waste or reconstituted waste fraction in each test container. Concentration series is based on volume of sample material since chemical analyses are not routinely conducted as part of the procedure.

Figure 5 presents an example laboratory data sheet for recording the test conditions and test result, i.e., pH - in high, medium, low test concentrations at zero time and at every 24 hours thereafter, dissolved oxygen - in high, medium, and low test concentrations. These parameters are measured at 0 time and every 24 hours thereafter, and temperature of a water bath or of controlled temperature area is recorded continuously or every 24 hours.

If no toxicity is detected at any concentration, and the 100 percent dose was tested, no further testing is required. The test material is considered to have no detectable toxicity and will be reported as such. The materials that kill or immobilize all or nearly all the test organisms should be retested with a lower dose range, so that an estimated EC_{50} can be determined.

c. tabulation of test result

An EC_{50} determination will be provided for the *Mysid* test. Binomial, Moving average, or Probit Analysis methods can be used to determine EC_{50} values. When the calculations are completed, a report of the test result should include the derivation of stock culture used during testing, the test conditions, and a mathematic and/or graphic interpretation of results expressed as EC_{50}.

TABLE 14. SUMMARY OF REQUIRED TEST CONDITIONS FOR *Mysidopsis bahia* TEST

Species	*Mysidopsis bahia*
Temperature, (use centigrade thermometer)	22-25°C (±2° of starting temperature)
Photoperiod, hours light:dark	Continuous ambient room light
Water quality (salinity)	32-36 ppt
Container size	1-liter container or 8-inch culture dish
Test container total volume	500 ml
Organisms per container	5
Replicates	3
Feed	Yes - approx. 20 *Artemia* (48 hours old) per *Mysid*.
Test duration	96 hours
Dissolved oxygen and pH measurement (using appropriate meters and electrodes)	0, 24 , 48, 72, 96 hours

V. Tabulation of Overall Test Result

The binomial method (Stephan, 1977), moving average technique (Stephan, 1977), or a probit analysis (Finney, 1971) can be used to calculate the EC_{50}. Many laboratories will also have a capability for computer assisted data tabulation. Computer availability will obviously allow for a more rapid comparison of sample results, and several computer programs for LD_{50}/EC_{50} determinations are currently available. However, it should be remembered that the bioassays have been conducted as screening tests only and have used a minimum number of test organisms.

While dose-response curves are prepared during each individual assay, the summary test result will be based on the EC_{50} and SC_{20} values. These results will be presented as shown in Table 15. As stated earlier, none of the four bioassays is conducted as a definitive test in which range finding evaluations precede assays that characterize the concentration response curve. The four component assays are screening procedures being used to test samples of unknown chemical composition. A minimum number of test organisms are used in a static

Data Record Sheet - *Mysidopsis bahia* Aquatic Bioassay

Test Sample:

Initiation: Date ____________________ Time ______________________________

Termination: Date ____________________ Time ______________________________

Test Temperature Range:

Time	0 h	24 h	48 h	72 h	96 h
Temp. (°C)					

Waste %	Test container number	Number of live organisms	Dissolved oxygen, mg/liter	pH	Conductivity
		0 24 48 72 96	0 24 48 72 96	0 24 48 72 96	0 24 48 72 96

Figure 5. Data Record Sheet - *Mysidopsis bahia* aquatic bioassay.

TABLE 15. PRESENTATION OF SUMMARY TEST RESULT FOR THE CHEMICAL FRACTIONATION/AQUATIC BIOASSAY PROCEDURE (Values Expressed as EC_{50} and SC_{20})

	Freshwater			Saltwater		
	Selenastrum		Daphnia	Skeletonema		Mysidopsis
Sample Type	EC_{50}	SC_{20}	EC_{50}	EC_{50}	SC_{20}	EC_{50}
Whole waste						
Total organics fraction						
Base/neutral subfraction						
Organic acid subfraction						
Organic residual subfraction						
Recombination of organic subfractions						
Total inorganics fraction						
Cation subfraction						
Anion subfraction						

test situation. Furthermore, chemical fractionation rarely results in absolute compound separation into the various fractions and subfractions. Fractionation efficiency will be based on the specific compounds present in the original sample material. However, the overall test result (Table 15) will provide relative information as to (1) which fraction(s) most likely contains the bioactive component(s), (2) what additive, synergistic, or antagonistic effects appear to be operating, and (3) what potential exists for stimulation of algal populations. The overall test should provide a good preliminary (or screening) hazard evaluation for the aquatic environment. In some instances, categories of relative toxicity might be substituted for actual LD_{50}/EC_{50} values. Tables 16 and 17 provide an example of this approach.

TABLE 16. EXAMPLE OF RELATIVE TOXICITY CATEGORIES THAT CAN SOMETIMES BE USED TO SUMMARIZE TEST RESULT

Assay	Test Results	Maximum Applicable Dose (%)[a]	Range of LC_{50} or EC_{50} Concentrations (%)			
			High	Moderate	Low	Not detectable
Freshwater invertebrate	48-h EC_{50}[b] (immobilization)	100	0-20	20-75	75-100	ND[c] at 100
Freshwater algae	96-h EC_{50} (growth inhibition)	100	0-20	20-75	75-100	ND at 100
Marine invertebrate	96-h EC_{50} (immobilization)	100	0-20	20-75	75-100	ND at 100
Marine algae	96-h EC_{50} (growth inhibition)	100	0-20	20-75	75-100	ND at 100

[a] Maximum applicable dose - maximum dose that can be prepared for the particular test system; or maximum dose likely to be encountered under environmental conditions. Maximum dose given as 100 percent.

[b] EC_{50} - calculated concentration expected to produce effect in 50 percent of population. The SC_{20} result for algal populations is not included in this table.

[c] ND - no detectable effect.

TABLE 17. EXAMPLE OF SUMMARY TABLE THAT PRESENTS LC_{50} RESULTS USING RELATIVE TOXICITY CATEGORIES
(High, Moderate, Low, and No Detectable Effect)*

	Freshwater			Saltwater		
Sample Type	Selenastrum		Daphnia	Skeletonema		Mysidopsis
Whole waste						
Organics fraction						
Base/neutral subfraction						
Acid subfraction						
Residual subfraction						
Recombination of organic subfractions						
Inorganics fraction						
Cation subfraction						
Anion subfraction						

* Test categories for stimulation of algal populations not included but could be prepared as was done for toxic effects (Table 16).

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